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UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

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PhD thesis 2019

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MARIA SØNDERGAARD THØFNER

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Title: A spontaneous model of central neuropathic pain - syringomyelia in the Cavalier King Charles Spaniel

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Cover page illustrations: Luxol-stained transverse section of Bertil Klagenberg's spinal cord segment C3 with asymmetric syringomyelia
Quantification of Luca Løvhøj's mechanical sensory threshold
Nellie and Chilli Hartmann in the sun
Sagittal T1W MRI of Molly Mikuta's neurocranium and cervical spinal cord
DNA string
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Preface

The present thesis has been submitted to complete the author's PhD programme, as required at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. The thesis is based on research undertaken at the Department of Veterinary Clinical Sciences, University of Copenhagen, and at the Core Center for Molecular Morphology, Section for Stereology and Microscopy; Center for Stochastic Geometry and Advanced Bioimaging, Department of Clinical Medicine, Aarhus University, from May 2013 to December 2018.

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Four original papers form the basis of this present thesis. They are referred to as Study I-IV in the text.

Paper I: Thøfner MS, Stougaard CL, Westrup U, Madry A, Knudsen CS, Berg HA, Jensen CSE, Handby RML, Gredal H, Fredholm M and Berendt M. Prevalence and heritability of syringomyelia in Cavalier King Charles Spaniels and long-term outcome in symptomatic and asymptomatic littermates. *J Vet Intern Med 2015; 29:243-250*

Paper II: Thoefner MS, Westrup U, Toft N, Bjerrum OJ, Berendt M. Mechanical sensory threshold in Cavalier King Charles spaniels with syringomyelia-associated scratching and control dogs. *Vet Journ 2019; 246:92-97*

Paper III: Thoefner MS, Jensen TS, Agerholm JS, Bjerrum OJ, Berendt M, Nyengaard JR. Superficial dorsal horn volume loss in Cavalier King Charles Spaniels with neuropathic pain and syringomyelia – a quantitative and qualitative histological characterisation of cervical spinal cord lesions. Manuscript in preparation

Paper IV: Thoefner MS, Skovgaard LT, McEvoy FM, Berendt M, Bjerrum OJ. Pregabalin alleviates clinical signs of syringomyelia-related central neuropathic pain in Cavalier King Charles Spaniels – a randomised controlled trial. *Manuscript submitted to Veterinary Anaesthesia and Analgesia, January 2019*

Vedbæk, Denmark, March 2019
Maria Søndergaard Thøfner, DVM

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Table of contents

List of abbreviations

CGRP	Calcitonin gene-related peptide
CKCS	Cavalier King Charles Spaniel
C1M	Chiari-1 malformation
CM	Chiari-like malformation
CM-SM	The Chiari-like malformation - syringomyelia complex in Cavalier King Charles Spaniels
CNeP	Central neuropathic pain
CSF	Cerebrospinal fluid
DN4	Douleur Neuropathique 4
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglion
fT_4	Free thyroxine, the metabolic active fraction of the thyroid hormone
GABA	Gamma-aminobutyric acid
GEE	Generalised estimating equation
GWAS	Genome-wide association study
IASP	International Association for the Study of Pain
LANSS	The Leeds Assessment of Neuropathic Symptoms and Signs
MRI	Magnetic resonance imaging
MST	Mechanical sensory threshold
MSTQ	Mechanical sensory threshold quantification
NMDA	N-methyl-D-aspartate
NPS	The Neuropathic Pain Scale
NPSI	The Neuropathic Pain Syndrome Inventory
NRS	Numeric rating scale
NSAID	Non-steroidal anti-inflammatory drug
PAG	Periaqueductal grey
PI	Principal investigator
PGN	Pregabalin
QST	Quantitative sensory test
SP	Substance P
STEP	Sensory threshold examination protocol
T1W	T1-weighted (MRI)
T2W	T2-weighted (MRI)
T_4	Thyroxine (tetraiodothyronine)
TCA	Tricyclic antidepressant
TRPV1	The capsaicin receptor - one of six vanilloid transient receptor potential ion channels
TSH	Thyroid-stimulation hormone
VAS	Visual Analogue Scale
WDR	Wide dynamic range neuron

Nomenclature

Allodynia	Pain due to a stimulus that does not normally provoke pain
Central neuropathic pain	Pain caused by a lesion or disease of the central somatosensory nervous system
Central sensitisation	Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input
Dysaesthesia	An unpleasant abnormal sensation, whether spontaneous or evoked. Special cases of dysesthesia include hyperalgesia and allodynia
Hyperalgesia	Increased pain from a stimulus that normally provokes pain
Hyperaesthesia	Increased sensitivity to stimulation. Includes both allodynia and hyperalgesia
Hypoaesthesia	Decreased sensitivity to stimulation
Hypoalgesia	Diminished pain in response to a normally painful stimulus
Neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system
Nociception	The neural process of encoding noxious stimuli
Nociceptive neuron	A central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli
Nociceptive pain	Pain that arises from actual or threatened damage to a non-neural tissue and is due to the activation of nociceptors
Nociceptive stimulus	An actually or potentially tissue-damaging event transduced and encoded by nociceptors
Nociceptor	A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli
Noxious stimulus	A stimulus that is damaging or threatens damage to normal tissues
Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage
Pain threshold	The minimum intensity of a stimulus that is perceived as painful
Paraesthesia	An abnormal sensation, whether spontaneous or evoked, that is not unpleasant
Peripheral neuropathic pain	Pain caused by a lesion or disease in the peripheral somatosensory nervous system
Peripheral sensitisation	Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields
Sensitisation	Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs

This table of terminology used in this thesis is a reproduction of the International Association for the Study of Pain’s Taxonomy (IASP 2012).

Summary

Symptomatic syringomyelia (SSM) is a spontaneous occurring neurological syndrome in Cavalier King Charles Spaniels (CKCS). Affected dogs present with physical and behavioural indicators of discomfort and pain characterised by spontaneous or evoked scratching, hypersensitivity to touch and paroxysmal pain manifestations with vocalisation. In human patients with SSM, 90% suffer pain, of these 40% experience neuropathic pain. The pathogenesis of syringomyelia is incompletely understood in both species and insufficient response to treatment is often seen. A lack of experimental animal models that mimic the clinical situation hinders development of effective analgesic compounds. The research presented in this thesis was undertaken to investigate if the CKCS with SSM represent a spontaneous model of central neuropathic pain (CNeP). The research questions of this thesis were investigated in four studies:

Study I: Prevalence and heritability of syringomyelia in Cavalier King Charles Spaniels and long-term outcome in symptomatic and asymptomatic littermates.

A cross-sectional study was undertaken to estimate the prevalence of SSM in the Danish population of CKCS born in 2001. Of 240 potential responders, 123 participated. Nineteen symptomatic dogs were identified after telephone interview validation. The classification of dogs as symptomatic was confirmed in 74% (14/19) after clinical examination. The estimated prevalence of SSM was 15.4% [9%, 22%]. To investigate the prevalence of SSM in families, eight symptomatic dogs from different litters were selected. The eight litters (34 siblings) comprised 17 asymptomatic and 17 symptomatic dogs. The incidence of symptomatic and asymptomatic dogs was converted to normal distributed mean liabilities, and their standard deviation from the threshold was used to estimate the heritability of SSM. A high heritability of 0.81 was found. This finding indicates that genetics have a strong impact on the total phenotypic variance in the population. An association between the expression of symptoms and magnetic resonance imaging (MR)-findings was investigated in the 34 littermates. Twenty-two dogs underwent clinical characterisation including MRI. The diameter of the syrinx and the syrinx/spinal cord ratio was significantly different between symptomatic and asymptomatic dogs ($P<0.01$). A significant association between the expression of symptoms and (1) syrinx diameter and (2) syrinx/spinal cord ratio was confirmed. Long-term outcome in symptomatic and asymptomatic dogs was assessed in 31/34 littermates (16 symptomatic and 15 asymptomatic). After five years, 93% (14/15) of asymptomatic dogs remained asymptomatic while 81% (13/16) of symptomatic dogs remained so. Progression of symptoms was seen in 31% (4/13) dogs. Four symptomatic dogs had been euthanised for ethical reasons. In conclusion, the clinical phenotype of middle-aged CKCS is static. When SSM has developed, it persists and may progress over time. Spontaneous recovery is less likely. The likelihood that older, asymptomatic syringomyelia-positive dogs develop symptoms is low.

Study II: Mechanical sensory threshold in Cavalier King Charles spaniels with syringomyelia-associated scratching and control dogs.

This prospective case-control study investigated, if the clinical phenotype is associated with mechanical sensory threshold (MST) alterations in CKCS with SSM. Nine CKCS with SSM and eight asymptomatic CKCS without syringomyelia were included. The MST was quantified with monofilaments on both sides of each dog's neck, and the individual dog's resulting MST was reported as a mean of the paired measurements. Unpaired comparison of log10-transformed mean MST between cases and controls was insignificant ($P=0.25$). Hence, it could not be confirmed that the clinical phenotype of SSM is characterised by mechanical threshold

alterations. Accordingly, it remains unknown whether symptomatic dogs have altered mechanical sensory thresholds

<u>Study III:</u> Superficial dorsal horn volume loss in Cavalier King Charles Spaniels with neuropathic pain and syringomyelia – a quantitative and qualitative histological characterisation of cervical spinal cord lesions.

This design-based stereological quantification and histopathological characterisation of spinal cord lesions was undertaken to investigate, if one or more specific structural cervical spinal cord entities involved in nociception were affected in CKCS with SSM. Spinal cord segments C1-C8 were investigated in eight cases and four controls. Seven of the eight included dogs with SSM expressed unilateral symptoms of CNeP. A stereological quantification of the total volume of the spinal cord segments C1-C8 and relevant sub-volumes was undertaken. Comparisons of the total mean volumes and sub-volumes revealed no significant difference between cases and controls. A significant volume loss of the dorsal horn laminae I-III was found on the affected side of the spinal cord from the seven dogs with unilateral symptoms (P=0.034). Hence, the relationship between unilateral symptoms of CNeP and a quantifiable ipsilateral structural loss of dorsal horn laminae I-III in CKCS with SSM was confirmed. Moreover, an association between lateralised symptoms and the structural grey matter volume loss was established. An additional unilateral degeneration of the dorsal root entry zone with dissection through pia mater was found in dogs with lateralised symptoms. The findings offer a structural explanation of the CNeP symptoms in CKCS with syringomyelia.

<u>Study IV:</u> Pregabalin alleviates clinical signs of syringomyelia-related central neuropathic pain in Cavalier King Charles Spaniels – a randomised controlled trial.

A randomised, double-blind, placebo-controlled cross-over trial was conducted to assess if pregabalin could function as an effective treatment of syringomyelia-related pain in CKCS. Thirteen dogs with SSM were included and randomised to treatment arm A (pregabalin → placebo; n=5) or treatment arm B (placebo → pregabalin; n=8). Treatment periods were of 25 days duration, segregated by a 48-hour wash-out period before cross-over. Outcome was number of scratching events during ten minutes of exercise at baseline and at four follow-ups. Eleven dogs completed the trial. The effect of pregabalin was estimated to be an 84% reduction from baseline in mean number of scratching events when compared to placebo (P<0.0001). Hence, the analgesic efficacy of pregabalin was found superior to placebo. Accordingly, pregabalin is an effective treatment of syringomyelia-related pain in CKCS.

This thesis provides novel information about neuropathic pain in CKCS with syringomyelia. Direct translation of symptoms of human neuropathic pain into animal models is probably unattainable. Nevertheless, CKCS with SSM constitute a promising counterpart to the experimental animal models in several aspects: the spontaneous development of disease, the slow progression over time and a diverse clinical phenotype. The histomorphological findings and the results of the clinical trial demonstrate the back- and forward translational potential. In conclusion, the author of this thesis proposes that the CKCS with SSM offers a superior, spontaneous model of neuropathic pain to fill the gap between the experimental animal models and human neuropathic pain patients.

Resumé (summary in Danish)

Symptomatisk syringomyeli (SSM) er en spontant forekommende neurologisk sygdom hos hunde af racen Cavalier King Charles spaniels (CKCS). Lidelsen er karakteriseret ved fysiske og adfærdsmæssige tegn på ubehag og smerte udtrykt ved spontan eller udløst kløe, over-følsomhed for berøring og anfaldsvise smerteudbrud med vokalisering. Smerte forekommer hos 90% af mennesker med SSM, heraf rapporterer 40% neuropatisk smerte. Forståelsen for udviklingen af syringomyeli hos både mennesker og hunde er ufuldstændig, og få individer opnår en tilfredsstillende effekt af smertebehandling. Udviklingen af nye, effektive smertestillende præparater er stagneret primært på grund af en mangel på eksperimentelle dyremodeller med symptomer, der afspejler menneskers symptomer. Denne afhandling præsenterer resultaterne af et forskningsprojekt, som har haft til formål at undersøge, om CKCS med SSM repræsenterer en spontan model for central neuropatisk smerte (CNeS). Afhandlingens forskningsspørgsmål blev undersøgt i fire studier:

<u>Studie I:</u> **Prevalence and heritability of syringomyelia in Cavalier King Charles Spaniels and long-term outcome in symptomatic and asymptomatic littermates**.

Prævalensen af SSM i den danske population af CKCS født i 2001 blev undersøgt i et tværsnitsstudie. Af 240 mulige respondenter svarede 123. Nitten symptomatiske hunde blev identificeret efter et bekræftende telefoninterview. Den korrekte klassificering af hunde som værende symptomatiske blev bekræftet i 74% (14/19) af hundene ved klinisk undersøgelse. Prævalens af SSM blev estimeret til 15.4% [9%, 22%]. Prævalensen af SSM i udvalgte familier blev estimeret efter udvælgelse af otte symptomatiske hunde fra forskellige kuld. De otte kuld (34 kuldsøskende) indbefattede 17 symptomatiske og 17 asymptomatiske hunde. Forekomsten af symptomatiske og asymptomatiske hunde blev omdannet til gennemsnit på den standardiserede normalfordeling, og gennemsnittenes standardafvigelse fra tærskelværdien blev anvendt til at estimere heritabiliteten af SSM. En høj heritabilitet på 0.81 blev påvist. Dette fund indikerer, at genetiske faktorer har en stor indflydelse på den totale fænotypevarians i populationen. Sammenhængen mellem forekomsten af symptomer og fund på magnetisk resonans skanning (MRI) blev undersøgt i de 34 kuldsøskende. Toogtyve hunde blev undersøgt klinisk og fik foretaget en MR-skanning. Der var en signifikant forskel i syrinx-diameter og syrinx/rygmarvs-ratio imellem symptomatisk og asymptomatiske hunde (P<0.01). Dermed blev sammenhængen mellem symptomer og (1) syrinx-diameter og (2) syrinx/rygmarvs-ratio bekræftet. Udviklingen af symptomer over tid blev undersøgt hos 31/34 kuldsøskende. Efter fem år var 93% (14/15) af de asymptomatiske hunde fortsat symptomfri, mens 81% af de symptomatiske hunde forblev symptomatiske. Hos 31% (4/13) forværredes symptomerne. Fire symptomatiske hunde var blevet aflivet grundet meget dårlig livskvalitet. Det kan konkluderes, at midaldrende hundes kliniske fænotype er statisk. Når SSM først er opstået, forbliver hunden symptomatisk, og sygdommen kan progrediere over tid. Spontan helbredelse er langtfra sandsynlig.

Studie II: Mechanical sensory threshold in Cavalier King Charles spaniels with syringomyelia-associated scratching and control dogs.

Dette prospektive case-kontrol-studie undersøgte, om den kliniske fænotype er associeret med ændringer i den mekaniske sensoriske tærskelværdi (MST) hos CKCS med SSM. Ni CKCS med SSM og otte asymptomatiske CKCS uden syringomyeli blev inkluderet. Den gennemsnitlige MST blev kvantificeret med monofilamenter på begge sider af den enkelte hunds hals, og den enkelte hunds MST blev beregnet som et gennemsnit af de parrede målinger. En uparret sammenligning af log10-transfomerede gennemsnits-MST var insignifikant

(*P*=0.25). Det kunne dermed ikke bekræftes, at den kliniske fænotype er karakteriseret ved ændringer i MST. Det forbliver derfor uvist, hvorvidt symptomatiske hunde har en ændret mekanisk sensorisk tærskelværdi.

Studie III: Superficial dorsal horn volume loss in Cavalier King Charles Spaniels with neuropathic pain and syringomyelia – a quantitative and qualitative histological characterisation of cervical spinal cord lesions.

Denne design-baserede stereologiske og histopatologiske karakterisering af rygmarvslæsioner blev foretaget med det formål at undersøge om syringomyeli afficerer én eller flere specifikke strukturelle enheder i rygmarven, som er involveret i nociception. Rygmarvssegmenterne C1-C8 fra otte cases og fire kontroller blev undersøgt. Syv af de otte inkluderede hunde med SSM udtrykte unilaterale symptomer på CNeS. En stereologisk kvantificering af totalvolumet af rygmarvssegmenterne C1-C8 og relevante sub-voluminer blev foretaget. De totale gennemsnitlige totalvoluminer og sub-voluminer var ikke signifikant forskellige mellem cases og kontroller. Der blev påvist et signifikant volumen-tab af dorsalhornets laminae I-III i den afficerede side af rygmarven hos de syv hunde med unilaterale symptomer (*P*=0.034). En sammenhæng mellem unilaterale symptomer og et kvantificérbart strukturelt tab af dorsalhornets laminae I-III hos CKCS med SSM blev dermed bekræftet. I tillæg blev unilateral degeneration af den Lissaurske tragt med dissektion gennem Pia Mater konstateret hos hunde med unilaterale symptomer. Dette fund fremsætter en strukturel forklaring på symptomer på CNeS hos CKCS med syringomyeli.

Studie IV: Pregabalin alleviates clinical signs of syringomyelia-related central neuropathic pain in Cavalier King Charles Spaniels – a randomised controlled trial.

Et randomiseret, dobbelt-blindet, placebokontrolleret overkrydsningsstudie blev udført for at vurdere, om pregabalin kan fungere som en effektiv behandling af syringomyeli-relateret smerte hos CKCS. Tretten hunde med SSM blev inkluderet og randomiseret til behandlingsarm A (pregabalin → placebo; n=5) eller behandlingsarm B (placebo → pregabalin; n=8). Behandlingsperioderne varede 25 dage og var adskilt af en 48-timers udvaskningsperiode før overkrydsning. Outcome-variablen var antallet af kløe-episoder i løbet af en ti minutters gåtur målt før studie-opstart og ved fire kontrolbesøg. Elleve hunde gennemførte behandlingsstudiet. Effekten af pregabalin blev estimeret til en 84% reduktion i antallet af kløe-episoder i forhold til før studie-opstart sammenlignet med placebo (*P*<0.0001). Den smertestillende effekt af pregabalin er dermed bedre end placebo. Pregabalin er altså fundet effektiv til at behandle syringomyeli-relateret CNeS hos CKCS.

Denne afhandling giver ny information om neuropatisk smerte hos CKCS med syringomyeli. Det er sandsynligvis uopnåeligt at udtrykke de præcise symptomer på neuropatisk smerte, som mennesker oplever, i dyremodeller. Ikke desto mindre udgør CKCS med SSM en lovende pendant til de eksperimentelle dyremodeller på flere punkter: den spontane udvikling af sygdom, den langsomme udvikling over tid og en uensartet klinisk fænotype. De histomorfologiske fund og resultatet af behandlingsstudiet viser potentialet for translation til både eksperimentelle modeller og mennesker. Slutteligt konkluderer forfatteren af denne afhandling, at CKCS med SSM vurderes som værende en fordelagtig spontan model for neuropatisk smerte, der kan binde bro mellem de eksperimentelle dyremodeller og mennesker med neuropatisk smerte.

Introduction

What is pain? Most of us would probably agree that pain is when it hurts. This statement is beautiful in its simplicity and, at least at first glance, straightforwardly understood. We imagine, we share a common understanding of what it is to experience pain. However, pain is in fact more complicated. How can veterinarians tell whether a dog suffers from headache? How can physicians understand the exact severity of pain experienced by their patients? And is a hoof abscess more painful to a horse than a bee sting on the shoulder of a child? Can we confirm or reject the presence of pain, can we measure pain and make inter-individual comparisons? The problem is that pain is subjective. Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Jensen et al. 2011). Yet, to describe any given experience with words can be challenging, to say the least, and to understand another subject's description of an experience is even more so.

Accordingly, there are no measurable biomarkers of pain. Human patients suffering pain can assess their experiences of pain on predefined pain scales. In experimental pain models, pain is measured in terms of a behavioural response to a given stimulus. There is no gold standard for the evaluation of pain within the veterinarian profession. Usually, a diagnosis of pain is based on the owner's observations and narrative, the clinical assessment and the animal's response to treatment (Mathews 2008).

Acute pain is a preserved trait across species and serves as a warning signal to protect an individual from harm. If acute pain persists beyond the expected time of recovery, it converts into a chronic state; the pain changes from being protective to become harmful (Kuner & Flor 2016). In humans, chronic pain has a major socio-economic impact and is a tremendous medical challenge. For example, 25-33% of the European and American population report chronic pain; hereof 7-20% predominantly experience pain with neuropathic characteristics (Breivik et al. 2006; Torrance et al. 2006; Bouhassira et al. 2008; Toth et al. 2009; van Hecke et al. 2014). The estimated prevalence of chronic pain in dogs is 17%, based on the only available cross-sectional study in 231 American university hospital outpatients (Muir et al. 2004). The diverse and often subtle clinical signs of chronic pain that dogs express probably means that there is an inadequate recognition of veterinary patients with chronic pain. This may result in an underestimation of the presence of pain and its implications for the individual animal's behaviour and quality of life. In addition, a thorough comprehension of the underlying mechanisms of chronic and neuropathic pain, the lack of licensed compounds and evidence-based treatment options may well result in undertreatment.

Neuropathic pain is pain caused by a lesion or disease of the somatosensory nervous system (IASP 2012). A sub-classification as either peripheral or central neuropathic pain depends on the localisation of the lesion. The aetiologies are numerous. The pain may arise as a consequence of functional changes in the somatosensory pathway, but this does not imply activation of nociceptors. However, not all patients with nervous system lesions develop neuropathic pain (Kehlet et al. 2006). To exemplify, central neuropathic pain (CNeP) occurs only in up to 50% of patients with traumatic spinal cord injury and in 40-70 % of patients with syringomyelia (Milhorat et al. 1996a; Siddall et al. 2003; Werhagen et al. 2004; Ducreux et al. 2006; Hatem et al. 2010). Syringomyelia-related neuropathic pain is fundamentally different from other types of pain. The pathogenesis is not fully understood and the pain phenotype is very diverse. In addition, an insufficient response to treatment is observed in most patients. There is thus an unmet medical need for effective analgesic compounds. Despite extensive preclinical research, we still lack experimental models that mimic the clinically relevant situations and the complex human neuropathic phenotype. A translational gap exists. This is due to an incomplete comprehension of the underlying mechanisms of central neuropathic pain together with insufficient predictive validity of the existing experimental models. The results are numerous failed clinical trials (Finnerup et al. 2015; Yekkirala et al. 2017).

Spontaneous symptomatic syringomyelia occurs with unusual frequency in dogs of the breed Cavalier King Charles Spaniel (CKCS)[I]. These patients represent a set of challenges to veterinarians that are just like those presented by their human counterparts to physicians. For this reason, the research project presented in this thesis was undertaken to provide novel information on canine neuropathic pain in CKCS with syringomyelia for the benefit of these dogs, their owners and their clinicians. Acknowledging this particular set of unmet medical needs, the overall aim of this thesis is to investigate if the CKCS with syringomyelia represent a spontaneous model of central neuropathic pain. The specific aims and hypotheses of the four conducted studies are respectively:

I. **To characterise the Danish CKCS population by estimating the prevalence and heritability of symptomatic syringomyelia, by investigating the association between clinical signs and MRI findings and by reporting the long-term outcomes in symptomatic and asymptomatic dogs.**

    H_A: There is an association between the expression of symptoms and (1) syrinx diameter and (2) syrinx / spinal cord ratio.

II. **To investigate if the clinical phenotype is associated with alterations in the cervical mechanical sensory threshold.**

    H_A: The structural spinal-cord parenchymal lesions result in a significant difference in mechanical sensory threshold between symptomatic CKCS with syringomyelia and asymptomatic CKCS without syringomyelia.

III. **To quantify and characterise the histopathological cervical spinal cord lesions and, on this basis, to investigate if one or more specific structural cervical spinal cord entities are affected by syringomyelia in CKCS with neuropathic pain.**

    H_A: There is a relationship between unilateral symptoms of central neuropathic pain and quantifiable structural loss of a specific anatomical cervical spinal cord entity in CKCS with syringomyelia.

IV. **To assess pregabalin's efficacy to reduce syringomyelia-related symptoms of central neuropathic pain**

    H_A: The analgesic efficacy of pregabalin is superior to placebo to reduce neuropathic pain symptoms in CKCS with syringomyelia.

[I] The author acknowledges that the term 'symptom' applies to any indication of a disease perceived by a patient. Accordingly, the term is in principle not applicable to animals. For brevity, the terms 'symptomatic' and 'asymptomatic' will be used in the present thesis to describe dogs that do or do not express clinical signs consistent with syringomyelia-related neuropathic pain.

Background

Neuropathic pain arises as a consequence of functional, biochemical and structural changes in the somatosensory pathway. To understand these functional changes, what follows now is an overview of the normal functions of the somatosensory system and the pathway of protective nociception. Subsequently follows a presentation of the clinical manifestations of neuropathic pain in humans before a summary of the current knowledge about the transition from the acute to the chronic pain state and on maladaptive changes in the somatosensory nervous system that result in neuropathic pain.

The pathway of sensation and nociception

Nociception describes the molecular and electrical events involved in the processing of a noxious stimulus, whereas pain is the subjective, conscious experience of nociception (IASP 2012). Under normal physiological conditions, innocuous and noxious stimuli are both detected by the peripheral terminals of primary afferent fibres, also known as free nerve endings. Innocuous touch, vibration and proprioception is detected by low-threshold mechanoreceptors. Innocuous thermal inputs are detected by thermoreceptors. Noxious mechanical, thermal and chemical stimuli are mediated through distinct subtypes of peripheral, polymodal nociceptors (Koch et al. 2018; Yam et al. 2018). A stimulus detected by a primary afferent's peripheral terminal induces an electrical impulse. The electrical impulse is propagated along the primary afferent's axon by activation of voltage-gated sodium channels. The impulse is conducted to the spinal cord dorsal horn via the dorsal root ganglia (DRG), where the primary afferent's cell body is located (Figure 1). In the dorsal horn, a subsequent activation of voltage-gated calcium channels results in presynaptic transmitter release and the consequent transmission of the peripheral stimulus into the central nervous system (Benarroch 2016; Yam et al. 2018).

The primary afferent axons enter the spinal cord in the dorsal root entry zone (DREZ) and terminate in the dorsal horns (Figure 1). There are three principal types of peripheral sensory fibres: Aβ-, Aδ- and C-fibres. The large-diameter Aβ-fibres are myelinated and thereby fast-conducting. Their activation threshold is low, which allows them to respond to vibration and light touch. The nociceptive Aδ- and C-fibres respond to noxious mechanical, thermal and painful stimuli. Aδ-fibres are medium-sized, thinly myelinated and hence their conduction velocity is intermediate. The small C-fibres are unmyelinated and slow-conducting. When a noxious stimulus is applied to the skin, the initial sharp and rapid-phase pain is mediated by Aδ-fibres, while C-fibres elicit the subsequent dull, burning, aching or itching pain (Ringkamp et al. 2013; Koch et al. 2018; Yam et al. 2018).

The dorsal horn is organised in five laminae originally described by Rexed (Rexed 1952). There are three main types of neurons in the spinal cord dorsal horn: projecting neurons, wide dynamic range (WDR) neurons and interneurons. The nociceptive Aδ- and C-fibres synapse with high-threshold nociceptive projecting neurons in lamina I and the outer layer of lamina II (lamina II_O). The touch and tactile stimuli with low-intensity are mediated through low-threshold Aβ- and C-fibres. These low-threshold, mechanosensitive fibres synapse with projecting neurons in the inner layer of lamina II (lamina II_i) in addition to the deeper layers III and IV (Benarroch 2016; Yam et al. 2018). The projection neurons situated in the deeper laminae are WDR neurons. Their free nerve endings have broad receptive fields, that is to say they innervate a larger area of the end-organ such as for example the skin. The WDR neurons receive both noxious and innocuous inputs from all three sensory fibre types. Hence, they respond to both touch and tactile stimuli in addition to noxious stimuli (Benarroch 2016). The projecting neurons transmit sensory input via the spinothalamic tract and other parallel pathways. A fundamental difference between WDR neurons and other types of neurons is the characteristics of their action potentials. A normal neuron fires 'all-or-none' action potentials when activated. The WDR neurons fire graded action potentials; this characteristic enables them to grade their response depending on the intensity and frequency of the peripheral stimulus. When WDR neurons are subject to repetitive stimulation, they respond by exaggerating their firing-rate. If the repetitive stimulation persists, the WDR neurons' membrane become increasingly susceptible to the afferent input. This sustained, partial depolarisation is also known as 'wind-up' (Voscopoulos & Lema 2010; Pozek et al. 2016). A third heterogeneous population of dorsal horn neurons are the inhibitory and excitatory interneurons. These interneurons modulate the projecting and WDR neurons' response before ascending transmission to higher centres of the somatosensory pathway. Inhibitory interneurons are predominantly gamma-aminobutyric acid (GABA)-ergic or glycinergic. Excitatory interneurons are glutaminergic (Benarroch 2016; Koch et al. 2018).

The axons of projecting neurons decussate the midline of the spinal cord and ascend in the direct sensory pathway via the spinothalamic tracts. The low-intensity touch and tactile stimuli are conveyed in the dorsal spinothalamic tracts. In contrast, innocuous thermal and noxious stimuli are transmitted in the lateral spinothalamic tract. The thalamus and periaqueductal grey (PAG) function as a relay station for inputs from the superficial laminae I and II_O. From here, the input is mediated to the somatosensory cortex, thus enabling a reflexive or adaptive response to the nociperception (Voscopoulos & Lema 2010; Dostrovsky & Craig 2013; Koch et al. 2018). The affective and emotional aspects of the pain experience is processed by interplay with the limbic system. The axons of the projecting laminae I and II_O neurons additionally ascend by the indirect sensory pathway via the spinoreticular tracts or synapse in brainstem nuclei, which contain descending projections to the dorsal horn (Dostrovsky & Craig 2013; Kuner & Flor 2016).

The descending pathway modulates the nociceptive signalling at several levels in the dorsal horns (Figure 1). The modulation occurs by interactions with peripheral afferent fibre terminals, projecting neurons, excitatory and inhibitory interneurons and terminals of other descending pathways. A battery of neurotransmitters, receptors and modulators are involved in this complex process. Noradrenaline released by descending efferents binds to ɑ-2 receptors of spinal projection neurons. The result is either direct inhibition of primary afferent transmitter release or indirect inhibition through acetylcholine release and activation of primary afferent inhibitory muscarinic M2-receptors. While serotonin is involved in both facilitation and inhibition, the endogenous opioids inhibit transmission by blocking the release of substance P (SP), the neurotransmitter released by primary afferents involved in nociception (Voscopoulos & Lema 2010; Treede 2016).

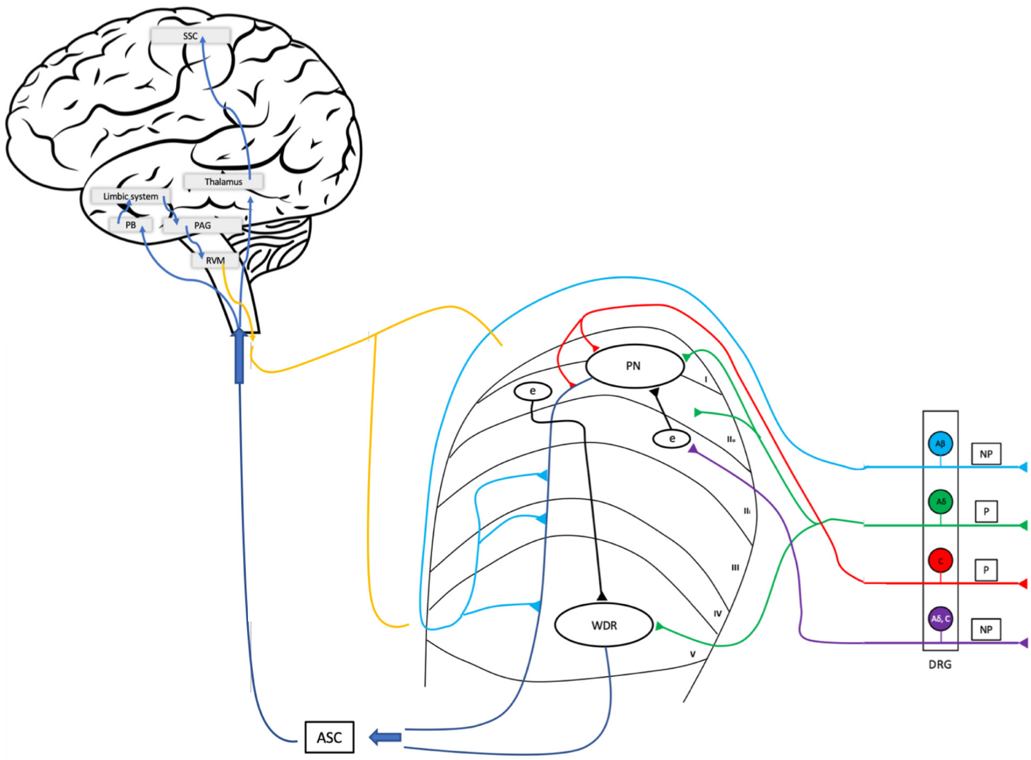


Figure 1 Schematic drawing of the pathway of sensation and nociception.

Innocuous and noxious peripheral stimuli are mediated through sensory Aβ-, Aδ- and C-fibres. The primary neurotransmitter of all the primary afferent fibres is glutamate. Yet, they can be sub-divided into two populations depending of the additional type of neurotransmitters that they release in the dorsal horn. The **peptidergic Aδ- and C-fibres** are indicated by **P** in the figure. These fibres contain substance P (SP) and calcitonin gene-related peptide (GCRP) and mediate noxious thermal stimuli. They synapse with projecting neurons (**PN**) in lamina I and interneurons in the outer layer of lamina II (lamina II_O). The **non-peptidergic Aβ-fibres** are indicated by **NP** in the figure. They synapse with neurons in laminae III-V and mediate innocuous vibration and light touch. **Non-peptidergic Aδ- and C-fibres** synapse with excitatory interneurons (**e**) in the inner lamina II (lamina II_i) and mediate noxious mechanical stimuli. These lamina II_i interneurons mediate the input to the projecting neurons in lamina I. The inhibitory (not shown in the figure) gamma-aminobutyric acid (GABA)-ergic and glycinergic interneurons, receive inputs from both Aβ-fibres and nociceptors. These inhibitory interneurons have three functionalities. First, they regulate the transmission of nociceptive inputs in the dorsal horn. In addition, they prevent activation of excitatory interneurons by input from the Aβ-fibres. Third, they control the excitability of projection neurons in lamina III-V of which some are wide dynamic range (**WDR**) neurons both receiving innocuous and noxious stimuli. The axons of projecting neurons ascend in the direct and indirect ascending pathway (**ASC**). The projecting neurons innervate the thalamus, parabrachial area (**PB**) and periaqueductal grey (**PAG**) from where the input is mediated to the somatosensory cortex (**SSC**), pathways that are also influenced by the limbic system. The descending pathway from the rostral ventromedial medulla (**RVM**) and other brain stem nuclei modulate the nociceptive process at several spinal levels. **DRG**: dorsal root ganglion. The diagram is a modified version of those given in (Latremoliere & Woolf 2009; Colloca et al. 2017).

Clinical manifestations of somatosensory nervous system lesions in neuropathic pain patients

Patients with pain due to a lesion or disease affecting the somatosensory nervous system typically present concomitant sensory abnormalities (Finnerup 2008; Watson & Sandroni 2016). The pain itself is chronic. It may be spontaneous ongoing or paroxysmal, meaning dominated by pain attacks. In other patients again, the pain can be evoked by innocuous or noxious stimuli (Gilron et al. 2015; Colloca et al. 2017). Pain evoked by a normally innocuous stimulus is designated as allodynia. Increased pain from a stimulus normally eliciting pain is designated as hyperalgesia (IASP 2012). Typically, the pain is located in an area with additional sensory abnormality. The sensory abnormalities are often characterised by a loss or gain of sensation (Gilron et al. 2015; Colloca et al. 2017).

An injury to the somatosensory nervous system causes neuronal loss, compromised transduction, conduction or transmission as a consequence of terminal atrophy, loss of axons or terminals respectively (von Hehn et al. 2012). This results in the development of a partial or complete sensory loss of sensation in the innervated area. The sensory loss can be specific or global and may affect one or all sensory modalities (innocuous or noxious mechanical and thermal stimuli) (Baron et al. 2010; Colloca et al. 2017). Hypoaesthesia designates a reduced sensitivity to normally innocuous stimuli; hypoalgesia designates diminished pain in response to a normally painful stimulus (IASP 2012).

Sensory gain arises as a consequence of disturbances in the normal homeostatic balance between excitation and inhibition. The inhibitory / excitatory imbalance can occur both in the peripheral and central nervous system. The nervous system's physiological balance converts into a hyper-excitable state, where neurons express an increased reaction to stimuli, both due to lowered detection thresholds and increased responses to stimuli. The result is allodynia and hyperalgesia (Gilron et al. 2015; Benarroch 2016; Colloca et al. 2017).

The maladaptive changes in the somatosensory system causing neuropathic pain

The structural, biochemical and functional changes after injury to the somatosensory nervous system and their implication on nociceptive processing have been extensively studied, especially in experimental animal models (Berge 2011; Koch et al. 2018; Kumar et al. 2018). However, how maladaptive changes are relevant in relation to the symptoms of neuropathic pain is still not adequately understood (Berge 2011; Bouhassira & Attal 2016; Kuner & Flor 2016). That is also the case for the different roles of peripheral and central mechanisms in neuropathic pain. Three key concepts have been put forward to explain allodynia and hyperalgesia, the sensory abnormalities that distinguish neuropathic pain from other chronic pain conditions: peripheral sensitisation, central sensitisation and central disinhibition (von Hehn et al. 2012; Gilron et al. 2015; Alles & Smith 2018). The concepts of sensitisation and disinhibition do not solely apply to the neuropathic pain state, but the pain triggers are different from other types of pain. To account for this in further detail, the following two subsections of this overview outline current knowledge about these key concepts, both in relation to acute tissue trauma and in the neuropathic pain state.

Peripheral sensitisation

Any tissue injury activates the arachidonic acid pathway and induces the release of inflammatory mediators. The cardinal signs of inflammation, calor, dolor, rubor, tumor and function laesa are results of the local inflammatory response due to increased vascular permeability and peripheral oedema (Voscopoulos & Lema 2010; Yam et al. 2018). The inflammatory mediators trigger the primary afferent's peripheral terminals to become more responsive to peripheral stimuli. In addition, silent nociceptors are activated. Silent nociceptors are a subgroup of nociceptors that are non-responsive under normal physiological conditions. The net result is an increased release of SP from the primary afferents and hence an increased afferent input to the spinal cord. This peripheral sensitisation is a physiological consequence of the original tissue injury. It serves to protect the tissue from further damage and facilitate healing by avoiding contact with and use of the injured tissue (Voscopoulos & Lema 2010; Yam et al. 2018). Classic examples of peripheral sensitisation are inflammatory pain after sunburn and post-surgery, where tissue injury results in spontaneous pain, allodynia and hyperalgesia. In the case of acute tissue damage, the above-mentioned adaptive changes in the function and properties of nociceptive neurons are reversible.

If a peripheral nerve is injured, the damaged axon releases i.a. nerve growth factor during degeneration. This triggers an upregulation of sodium channels and peripheral receptors, especially the vanilloid receptor TRPV1 on the remaining, intact primary afferents (von Hehn et al. 2012; Alles & Smith 2018). A concomitant loss of potassium channels increases the excitability of the primary afferents: the nociceptive threshold is reduced and their responsiveness to peripheral stimuli increases. In addition, action potentials are generated independent of outer stimuli. So-called ectopic activity develops in parallel to reduced nociceptive thresholds to mechanical, thermal and chemical stimuli (von Hehn et al. 2012; Gilron et al. 2015; Alles & Smith 2018). This peripheral hyper-excitable state mimics the situation of acute tissue damage, where hyperalgesia develop within minutes after the inflicted injury (Yam et al. 2018). In contrast with acute tissue damage, the adaptive changes in the function and properties of the nociceptive neurons are most often irreversible in the neuropathic pain state (Kuner & Flor 2016).

Central sensitisation and disinhibition

The increase in peripheral activity described above has an impact on the central nervous system's processing of inputs at multiple sites. Presynaptic release of SP and increased peripheral spontaneous activity both enhance the dorsal horn's modulation of peripheral inputs. The result is a prolonged response (Gilron et al. 2015; Benarroch 2016). In addition, the WDR neurons, which normally only respond to inputs from nociceptive primary afferents, become responsive to inputs from the Aβ-fibres when these detect peripheral light touch and punctate stimuli (Benarroch 2016). A post-synaptic activation of the N-methyl-D-aspartate (NMDA)-receptor occurs by release of glutamate. In turn, the NMDA-receptor activation results in a cascade of pre- and postsynaptic upregulation of receptor- and neurotransmitter expression. T-type calcium channels are activated, and an increased α2δ-subunit expression occurs. The α2δ-subunit-upregulation facilitates an elevation of intracellular Ca^{2+}, and a concomitant upregulation of sodium channels causes long-term potentiation and amplification of peripheral inputs (Prescott et al. 2014; Kuner & Flor 2016; Alles & Smith 2018). The net result is an increased excitability of the dorsal horn neuronal. Increased thalamic neuronal excitability and the activation of microglia and astrocytes are additional contributors to the amplification of central excitatory signalling in central sensitisation (Gwak et al. 2017).

This state of central sensitisation in neuropathic pain is also characterised by reduced inhibitory modulation. This reduced inhibition may occur due to a loss of inhibitory interneurons or a phenotypic switch in GABAergic neurons from inhibitory to excitatory (Guo & Hu 2014; Gilron et al. 2015). In addition, an impaired central descending inhibition - characterised by attenuated noradrenergic inhibition and enhanced serotonin signalling - may contribute to central gain and maintenance of the pain state (von Hehn et al. 2012). The peripheral and central mechanism may become entangled into a vicious circle; recent findings indicate that central lesions after spinal cord injury subsequently activate peripheral pain mechanisms, which then contribute further to the sensory abnormalities in neuropathic pain patients (Carlton et al. 2009; Tankisi et al. 2015).

Assessing and diagnosing neuropathic pain in humans

To classify pain as being neuropathic, the following diagnostic criteria should be met: First, there should be a history of a potential or previously encountered nervous system lesion or disease. Second, the patient should report pain and sensory abnormalities with neuropathic characteristics and a neuroanatomical distribution that correlates with the nervous system lesion or disease. And, finally, a clinical examination including sensory assessment should be conducted to confirm the presence or absence of specific sensory abnormalities, allodynia and hyperalgesia. In the case of pain and sensory abnormalities of unknown aetiology, an objective confirmation of a lesion or disease should be sought by means of e.g. magnetic resonance imaging (MRI), tissue biopsies or electrophysiological tests to clarify the specific symptoms and distribution of somatosensory dysfunction (Cruccu et al. 2010; Finnerup et al. 2016).

Screening and assessment tools

Different screening tools, for example LANSS (the Leeds Assessment of Neuropathic Symptoms and Signs), DN4 (Douleur Neuropathique en 4 questions) and PainDETECT can be applied in the initial clinical work-up to distinguish between neuropathic and non-neuropathic pain (Bennett 2001; Bennett et al. 2005; Bouhassira et al. 2005; Freynhagen et al. 2006; Attal et al. 2018). The assessment tools NPS (the Neuropathic Pain Scale) and NPSI (the Neuropathic Pain Syndrome Inventory) are used to characterise the pain phenotype, to assess the changes over time and to evaluate treatment effects (Attal et al. 2018). The visual analogue scale (VAS) and the numeric rating scale (NRS) are used for self-reported pain intensity, and the latter is incorporated in the NPS and NPSI. (Cruccu et al. 2010; Attal et al. 2018). Despite a correct classification in 80-90% of cases, questionnaires cannot and should not replace clinical evaluation of pain patients (Bouhassira & Attal 2016; Attal et al. 2018).

Sensory assessment

Evaluation of the somatosensory profile includes assessment of the various sensory modalities using mechanical and thermal stimuli (Cruccu & Truini 2009). The evaluation can be based on an all-or-non-response, a graded response or by a comparison of sensations between a test and reference site. Table 1 presents an overview of some of the qualitative and quantitative assessment tools that are available. Qualitative assessment is undertaken in the clinical setting, whereas the experimental situation often requires quantification of thresholds before enrolment in a clinical trial. The sensory assessment will confirm the presence or absence of negative and positive sensory signs as a consequence of gain or loss. Moreover, the results are used to map the body areas that are affected by somatosensory dysfunction and to describe, which specific sensory modalities that are affected. The results help the physician to determine the grade of loss or gain and to assess the relevance of the findings in relation to the underlying cause of the neuropathic pain. Treatment can be initiated based on the aetiology of neuropathic pain, or the patient's somatosensory phenotype, or a combination of both (Jensen & Finnerup 2014; Colloca et al. 2017).

Table 1 Tools for qualitative and quantitative sensory assessment of somatosensory dysfunction.
A schematic presentation of the qualitative (bedside) and quantitative assessment tools for the evaluation of mechanical and thermal sensitivity in neuropathic pain patients.

	Assessment tool		
	Qualitative	Quantitative	QST outcome
Mechanical sensitivity			
Cutaneous punctate			
- blunt	Blunt-tiped probe	Monofilaments	N/A
- sharp, pin prick	Wooden stick	Weighted needles	Mechanical pain threshold
Deep pressure	Finger pressure	Pressure algometer	N/A
Dynamic mechanical - light touch	Cotton wool, paint brush	Monofilaments, SENSElab brush*	Mechanical detection threshold
Vibration	Tuning fork	Vibrameter	Vibration threshold
Thermal sensitivity			
Innocuous cool	Thermoroller, 20°	Graded thermal stimuli	Thermal cold detection threshold
Innocuous warm	Thermoroller, 40°	Graded thermal stimuli	Thermal warm detection threshold
Noxious cold	Thermoroller, 20°	Graded thermal stimuli	Thermal cold pain threshold
Noxious heat	Thermoroller, 40°	Graded thermal stimuli	Thermal heat pain threshold

QST, quantitative sensory test. *SENSElab 05, Somedic, Hörby, Sweeden. The table is a modified version of those given in (Walk et al. 2009; Jensen & Finnerup 2014).

Pharmacotherapy of central neuropathic pain

Neuropathic pain is challenging to manage. As opposed to inflammatory and other types of chronic pain, the effect of non-steroidal anti-inflammatory drugs (NSAIDs) and opioids is limited in neuropathic pain conditions (Finnerup et al. 2015; Yekkirala et al. 2017). A sufficient pain relief is seen in less than 60% of patients. Most neuropathic pain patients suffer pain of moderate severity despite treatment, and increased doses result in adverse effects rather than better pain relief (Finnerup et al. 2015). Systematic reviews and meta-analysis of randomised, double-blind studies on pharmacotherapy for neuropathic pain frame the available evidence-based guidelines (Attal et al. 2010; Finnerup et al. 2015). The treatment recommendations incorporate clinical efficacy, tolerability and safety, ease of use and costs. First-line treatment options are tricyclic antidepressants (TCAs), serotonin-noradrenaline reuptake inhibitors and gabapentinoids (Finnerup et al. 2015).

In general, the efficacy of treatment is less dependent on the neuropathic pain aetiology compared to the underlying mechanisms. Increasing evidence suggests that understanding the pain phenotype on the basis of a sensory profile increases the likelihood of success (Max 1990; Woolf et al. 1998; Attal et al. 2011). However, a central clinical challenge is the lack of bedside assessment tools to determine the underlying mechanism of any individual patient's particular pain phenotype. In addition, several symptoms may arise due to one specific mechanism. Finally, most of the active compounds used in the current treatment strategies target several entities in the nociceptive pathway rather than being selective towards one single mechanism (Alles & Smith 2018).

Syringomyelia in humans

The term syringomyelia was originally introduced by D'Angers in 1827 to describe what was later recognised as the naturally occurring central canal of the spinal cord (d'Angers & Prosper 1827). Today, the term is used to describe fluid-filled cavities within the spinal-cord parenchyma (Levine 2004; Roser et al. 2010; Magge et al. 2011; Blegvad et al. 2014; Klekamp 2018; WHO 2019). Syringomyelia may occur as a consequence of spinal malformations, extradural, intra- or extramedullary tumours, trauma, inflammation, haemorrhage, ischemia, or degenerative disc disease. In addition, syringomyelia is found concomitant to a variety of developmental skull anomalies associated with a reduced volume of the posterior fossa of which Chiari-1 malformation (C1M) is the most prevalent (Tubbs 2015; Klekamp 2018). The in vivo diagnosis requires MRI (Figure 2). Due to inconsistencies in the definition of syringomyelia, the prevalence in the general population is unknown but may be as low as 8.2-8.4 per 100.000 in Western countries (Brewis et al. 1966; Brickell et al. 2006; Kahn et al. 2015). However, syringomyelia is present in 45-73% of symptomatic C1M-patients (Milhorat et al. 1999; Sakushima et al. 2012; Strahle et al. 2015; Dlouhy et al. 2017; Klekamp 2018).

Chiari-1 malformation

The diagnosis of C1M is based on sagittal T1W MRI and is defined by a caudal cerebellar tonsil displacement of ≥5 mm below the foramen magnum (Aboulezz et al. 1985; Azahraa Haddad et al. 2018). Asymptomatic C1M is reported in 0.5-4.3% of people (Meadows et al. 2000; Vernooij et al. 2007; Aitken et al. 2009; Morris et al. 2009; Strahle et al. 2011; Evans 2017). The estimated prevalence of symptomatic C1M is 1% (Aitken et al. 2009). Different types of headaches are reported in 80-100% of C1M patients, most often paroxysmal intense headache of short duration (Milhorat et al. 1999). Moreover, patients often report tinnitus and symptoms related to compression of the brainstem and displacement of the cerebellum, namely visual and sleep disturbances and balance problems (Batzdorf 2015; Langridge et al. 2017).

Figure 2 MRI characteristics of human syringomyelia.

T1W sagittal MRI of a 64-year old female with Chiari-1 malformation, cerebellar tonsillar herniation and holocord syringomyelia characterised by hypointense (black) appearance of a fluid-filled cavity in the entire length of the spinal cord. Reproduced with permission (Muthukumar 2012).

Aethiopathogenesis and classification of syringomyelia

Despite extensive clinical and experimental research including theoretical and mechanical models, the underlying pathogenesis of syrinx formation remains unknown (Greitz 2006; Koyanagi & Houkin 2010; Linninger et al. 2016). With emerging novel techniques enabling visualisation of CSF flow dynamics, the most recent suggested pathogeneses and subsequent classification focus either on the alterations in CSF flow dynamics or on compromised extracellular fluid absorption due to obstructions in the atlanto-occipital space (Oldfield et al. 1994; Levine 2004; Greitz 2006; Williams 2008; Koyanagi & Houkin 2010). However, morphometric studies have failed to confirm a relationship between the presence of syringomyelia and the size of the posterior fossa in spite of a significantly reduced posterior fossa volume in paediatric C1M-patients when compared to controls (Vega et al. 1990; Milhorat et al. 1999; Trigylidas et al. 2008). In addition, the degree of tonsillar herniation is not correlated with the presence and size of the syrinx in humans with C1M-associated syringomyelia (Stovner & Rinck 1992; Milhorat et al. 1999). None of the proposed classifications have gained international consensus. In itself, this demonstrates the complexity of the disease: syringomyelia is found concomitant with a variety of other disorders, in some instances the aetiology is unknown, the pathogenesis is still not fully understood and the concept of syringomyelia therefore cannot be explained by one, coherent hypothesis.

Clinical characteristics of Chiari-1 malformation associated syringomyelia

Patients with C1M-associated syringomyelia present a variety of symptoms dependent on the localisation and extension of the syrinx. The clinical course is progressive and the symptoms are of cranial or spinal origin, reflecting the C1M or/and the spinal cord lesions (Todor et al. 2000). Common complaints of spinal origin are somatosensory deficits with or without pain. The type and degree of symptoms varies from subtle and diffuse to pronounced sensory abnormalities due to a temporal and spatial progression of the lesion (Cohodarevic et al. 2000; Ducreux et al. 2006; Hatem et al. 2010). Over time patients may develop concomitant motor-impairment, segmental weakness, muscle atrophy and autonomic dysfunction. The clinical-neurological examination may reveal trophic skin changes, scoliosis, uni- or bilateral reduced muscle strength, muscle atrophy and hyporeflexia in the upper extremities. As the disease progresses, arthropathy due to denervation may evolve, as may ataxia in the lower extremities, upper motor neuron signs (hyperreflexia) and spasticity may evolve (Cohodarevic et al. 2000; Levine 2004).

Pain is the major complaint in up to 90% of adult patients with C1M-associated syringomyelia (Barbaro et al. 1984; Cohodarevic et al. 2000; Todor et al. 2000; Klekamp 2018). Of these, up to 40% experience neuropathic pain (Milhorat et al. 1996a). Typically, the pain is present in an area with concomitant sensory abnormality in combination with hypo- or hypersensitivity or it may be dissociative, typically with a loss of thermal and pain sensation and preservation of touch sensation. The somatosensory abnormalities are segmental and extends over several dermatomes dependent on the syrinx-distribution. Usually, the somatosensory abnormalities are localised to the cervicodorsal dermatomes with a uni- or bilateral, symmetric or asymmetric distribution (Cohodarevic et al. 2000; Ducreux et al. 2006; Hatem et al. 2010). Coughing and sneezing, physical activity and posture change, stress and barometric fluctuations can intensify the pain (Cohodarevic et al. 2000). The neuropathic pain is either spontaneous ongoing (stimulus-independent), paroxysmal or evoked. Common descriptors are radicular, burning, dull, aching, pressing, throbbing or stretching. The abnormal sensations may be mild paraesthesia, dysaesthesia characterised by tingling, stinging, pins and needles or stimulus-evoked pains including brush, cold or pressure allodynia and mechanical or thermal hyperalgesia. The intensity varies, and a combination of characteristics are often reported (Milhorat et al. 1996a; Cohodarevic et al. 2000; Todor et al. 2000; Ducreux et al. 2006; Hatem et al. 2010).

Neurohistopathology

The most extensive report on clinical, autopsy and neuropathological findings in 175 syringomyelia cases is the publication by Milhorat *et al*. (2001). Based on the pathological findings and MRI-confirmed clinicopathological correlates the authors classify syringomyelia as 1) communicating syringomyelia characterised by central canal dilations communicating with the 4th ventricle; 2) non-communicating syringomyelia characterised by either primary parenchymal cavitations secondary to spinal cord trauma, ischemia or haemorrhage or by central canal / paracentral syringes associated with *inter alia* C1M, spinal arachnoiditis or extramedullary compression; 3) atrophic cavitations due to injury-induced parenchymal loss without a cerebrospinal fluid (CSF) filling mechanism (syringomyelia ex vacuo) and 4) neoplastic cavitations.

C1M-related syringomyelia in humans is usually non-communicating with the 4th ventricle (Milhorat et al. 1995). Hence, one or more rostral cervical segments are syrinx-free. In addition, a patent central canal lined with intact ependyma is overt. When a syrinx emerges in more caudal cervical segments, the central canal is initially not involved in syrinx formation. Later, moving further caudally, a fusion between the syrinx and the central canal is often seen. The syrinx diameter is usually widest in the cervical segments and tapers caudally. The lining of the cavities varies from thick connective tissue, glial infiltration, disrupted ependyma to a disintegrating rarefaction of grey or white matter. Neovascularisation and vascular changes characterised by a dilated lumen and thickened adventitia is seen within the syrinx and in the adjacent parenchyma (Milhorat et al. 1995).

The syrinx shape is often complex, and multiple fluid-filled cavities are occasionally reported (Hinokuma et al. 1992; Milhorat et al. 1995). The grey matter is primarily affected by the cavitation with a resultant neuronal loss. When white matter is affected by cavitation, dorsal white matter hypotrophy is most frequently reported. At the level with the most prominent syrinx expansion, the syrinx shape is usually slit-like with uni- or bilateral degeneration of dorsal horn grey matter with or without communication to the subarachnoid space. In five cases with unilateral symptoms, degeneration of the DREZ has been described in the ipsilateral, relevant half of the spinal cord (Netsky 1953; Milhorat et al. 1995; Beuls et al. 1996).

One case-control study has subjectively assessed the distribution of the peptidergic neurotransmitter SP in the spinal cord dorsal horns in ten patients with syringomyelia and ten patients without neurological disease (Milhorat et al. 1996b). As previously described, SP is primarily found in dorsal horn terminals of primary afferents involved in nociception. The distribution of SP was even in segments rostral to the syrinx between cases and controls. In segments affected by syringomyelia, a marked reduction or absence of SP was seen in 9/10 spinal cords. In segments immediately caudal to the syrinx, an increase in SP-immunoreactivity was seen in dorsal horn laminae I, II, III and V. This finding was ascribed to an increased number of SP-containing vesicles and terminal-like processes extending up to five dermatomes caudal to the syrinx. In cases with asymmetric syrinx distribution, a tendency to increased dorsal horn SP-immunoreactivity was seen in the ipsilateral dorsal horn (Milhorat et al. 1996b).

Syringomyelia in dogs

As in humans, syringomyelia develops secondary to inflammation, trauma, obstruction of CSF flow due to space-occupying lesions and disorders of the cranial caudal fossa and cranio-cervical junction in dogs. Syringomyelia has been reported in several different breeds (Child et al. 1986; Kirberger et al. 1997; Levitski et al. 1999; Taga et al. 2000; da Costa et al. 2004; Dewey et al. 2004; Takagi et al. 2005; Jung et al. 2006; MacKillop et al. 2006; Scrivani et al. 2007; Oxley & Pink 2012). The most prevalent concomitant MRI finding in dogs with syringomyelia is Chiari-like malformation (CM). The CM-associated syringomyelia (CM-SM) complex is inherited in the CKCS and Griffon Bruxellois (Rusbridge & Knowler 2004; Rusbridge et al. 2009). The following paragraphs present the available published material on the CM-SM complex in the CKCS up until the initiation of the research project presented here.

CM-SM was reported in the CKCS for the first time in 1997 (Rusbridge 1997). In the following years, the existing sparse case reports predominantly focused on diagnostic work-up in symptomatic cases (Churcher & Child 2000; Rusbridge et al. 2000; Lu et al. 2003). Asymptomatic syringomyelia was occasionally reported as an incidental finding (Lu et al. 2003; Couturier et al. 2008). In succeeding years, an increased number of symptomatic cases triggered an awareness of potential breeding implications in this very popular breed. In response, MRI screening for breeding purposes was initiated in the United Kingdom in 2006 (Cappello & Rusbridge 2007).

Estimates of asymptomatic syringomyelia are based on screening MRI for breeding purposes. In a retrospective assessment of 555 pre-breeding screening MRIs of CKCS from the United Kingdom and the Netherlands, the prevalence of asymptomatic syringomyelia of 46% has been reported based on owner-assessed absence of clinical signs (Parker et al. 2011). The occurrence of asymptomatic syringomyelia increases with age; from 25% in dogs up to 12 months of age to 70% in dogs ≥ six years of age. CM is found in more than 95% of MRI scanned CKCS independently of the reason for MRI and of the presence or absence of syringomyelia (Lu et al. 2003; Couturier et al. 2008; Carrera et al. 2009; Loderstedt et al. 2011).

Pedigree database analysis of over 1300 breeding dogs registered in the British Kennel Club has indicated a higher incidence of syringomyelia and concomitant CM in certain families and lines with one common, female ancestor (Rusbridge & Knowler 2003). A subsequent analysis of heritability based on MRI findings in 384 dogs, independently of the presence or absence of symptoms, reported a moderate genetic effect of 0.32-0.37 with regard to susceptibility of developing syringomyelia (Lewis et al. 2010). Pedigree analyses indicate a polygenic or complex origin with a variable penetrance, that is to say, the breeding of two syringomyelia-negative parents may result in both syringomyelia-positive and -negative offspring in the same litter (Lewis et al. 2010).

Clinical characteristics of Chiari-like malformation associated syringomyelia

Clinical signs of CM-SM can present at any age (Rusbridge et al. 2006). However, dogs with more severe clinical signs are often young and, in that case, the disease progression is often faster compared to dogs where clinical signs appear later in life (Rusbridge 2005; Rusbridge et al. 2006). The characteristics of the CM-SM phenotype includes both physical and behavioural indicators of pain (Rusbridge et al. 2007; Cerda-Gonzalez et al. 2009; Rutherford et al. 2012). In some cases, concomitant neurological deficits, for example proprioceptive or lower motor neuron signs, primarily affecting the thoracic limbs are present (Rusbridge et al. 2000).

The most prevalent clinical sign is intermittent spontaneous or evoked scratching with skin contact yet without any clinically overt underlying cause (Rusbridge et al. 2000; Rusbridge et al. 2006). The scratching is uni- or bilateral and is directed at the head, ears, neck, shoulders, axillae, chest or ventral abdomen. Factors that aggravate the scratching are collars and harnesses, bathing in warm or cold water, excitement and stressful situations. The intensity of scratching may increase in intact bitches during oestrus (Plessas et al. 2012). "Phantom scratching", a scratching reflex without skin contact, is also a descriptor of the clinical CM-SM phenotype (Rusbridge et al. 2000; Rusbridge et al. 2006). Another common clinical sign is hypersensitivity and reluctance to be touched on the head, neck, shoulder, sternal area and extremities. Some dogs attempt to escape wearing collars or harnesses. Others resist being showered and groomed. Paroxysmal pain manifests in severe cases with vocalisation, intense scratching, rubbing and circling on the ground (Rusbridge & Jeffery 2008; Cerda-Gonzalez et al. 2009) .

Pain is reported in up to 35% of symptomatic dogs (Todor et al. 2000; Rusbridge et al. 2007). Palpation may elicit signs of hyperaesthesia, primarily in the face, on the neck, sternal and spinal area, and a pain response may be elicited by cervical palpation or manipulation. Owners often report behavioural changes characterised by sleeping difficulties, restlessness, hypersensitivity to noise, strong sunlight and wind (Rusbridge et al. 2006). In addition, a strong association has been demonstrated between predefined indicators of neuropathic pain and stranger-directed fear, non-social fear, separation-related behaviour, attachment behaviour, excitability and pain sensation (Rutherford et al. 2012).

Diagnostic imaging

MRI is the gold standard for diagnosing syringomyelia (Figure 3). In the CKCS, syringomyelia is defined as a fluid-filled cavity within the spinal-cord parenchyma with a diameter of ≥ 2 mm on T1W MRI (Cappello & Rusbridge 2007). Syringomyelia has a predilection for the cervical spinal cord segments C2-C4 (75 % of cases), but dogs with cervical syringomyelia also present with thoraco-lumbar (76%) or lumbar syringomyelia (49%) (Loderstedt et al. 2011). The clinical manifestations of syringomyelia and the degree of pain are linked to the syrinx diameter relative to that of the spinal cord and to the lack of syrinx symmetry. The greater the syrinx/spinal cord ratio, and the more asymmetrical distribution directed towards the dorsal horns on the transverse images, the more severe are the symptoms and signs of pain (Rusbridge et al. 2007).

The implication of CM is identified on MRI as cerebellar indention or partial cerebellar herniation into or through the foramen magnum (Cappello & Rusbridge 2007). A smaller ratio of the caudal fossa volume relative to the total cranial cavity volume is associated with the presence of clinical signs. In addition to CM and syringomyelia, the MR images often reveal concomitant kinking of the spinal cord in the cranio-cervical junction, ventriculomegaly and otitis media with effusion. However, due to the very diverse clinical phenotype, it is difficult to investigate the correlation between the presence and severity of clinical signs and findings from diagnostic imaging. Consequently, the clinical significance of CM, whether on its own or combined with otitis media with effusion, is subject to continuous debate (Lu et al. 2003; Cerda-Gonzalez et al. 2009; Driver et al. 2012).

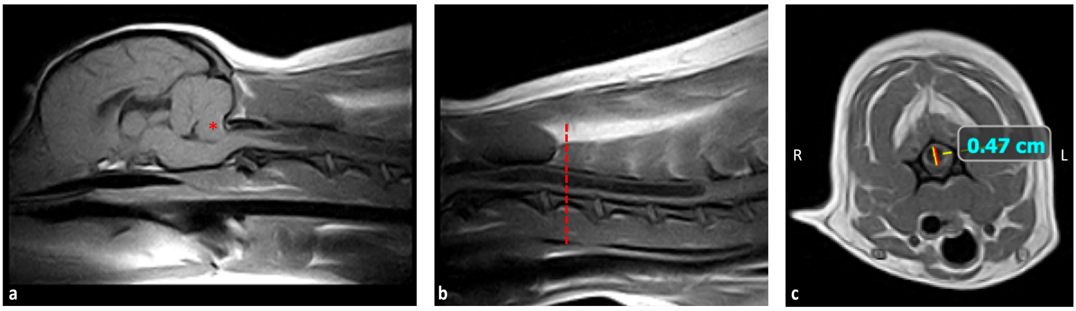


Figure 3 MRI characteristics of canine syringomyelia.
T1W sagittal (**a** and **b**) and transverse (**c**) MRI of a 21-months old spayed female Cavalier King Charles Spaniel with lateralised scratching directed at the right (**R**) side of her neck. MRI-findings were Chiari-like malformation and partial cerebellar herniation indicated by a red asterix in (**a**) and asymmetric (right) cervical syringomyelia characterised by hypointense (black) appearance of a fluid-filled cavity in (**b**) from the cranial level of the spinal cord's segment C1 to the cranial level of C5. The height of the syrinx was 0.47 cm, and the syrinx/spinal cord ratio was 0.66 measured on the transverse T1W image (**c**) where the syrinx was widest. The dotted, red vertical line in (**b**) marks the position of the transverse image (**c**). Reprint with permission from the dog's owner.

Clinical pain assessment in dogs

There is no gold standard for pain assessment in dogs. Consistent with the assessment of pain in human patients, the history and clinical examination is a central part of the diagnostic approach. As indicated by the clinical signs described above, the clinical manifestations of discomfort and pain in dogs are both physical and behavioural. Owners' description of the dog's normal behaviour and their description of the dog's aberration from such behaviour is very important information in the overall assessment. The veterinarian's challenge is to assess to what extent the reported aberrant behaviours and often subtle clinical signs of discomfort are linked to pain.

Several tools have been developed for the assessment of acute postoperative pain, chronic inflammatory pain and quality of life. The degree of validation varies, and the use is restricted to specific pain-related disorders, e.g. osteoarthritis (Wiseman-Orr et al. 2004; Reid et al. 2007; Hielm-Bjorkman et al. 2009). Application of thermal and mechanical stimuli has been used in quantitative sensory assessments in healthy dogs, in dogs with acute pain to assess the analgesic effect of systemic ketamine, opioids and local analgesics and in dogs with persistent pain due to cruciate ligament rupture (Duque et al. 2004; KuKanich et al. 2005a; KuKanich et al. 2005b; Fitzpatrick et al. 2010; Case et al. 2011; Hardie et al. 2011; Pieper et al. 2011; Brydges et al. 2012). However, the somatosensory function and the relationship between sensory threshold and behavioral indicators of pain in dogs with anticipated CNeP have not been assessed prior to the inception of the present research project.

Neurohistopathology

Prior to the work presented in this thesis, reports on the neuropathology associated with syringomyelia in the CKCS have been very sparse. One study has described the histopathological changes in six asymptomatic and six symptomatic syringomyelia-positive CKCS (Hu et al. 2012b). The description of syrinx extension and morphology was based on sections from either the C3 segment, a thoracic or a lumbar segment. The central canal was involved in syrinx formation in all but one case. The syringes were predominantly centred in the dorsal quadrants, 50 % were asymmetric and primarily affected the dorsal horn. The cavity margins were either lined by astrocytes and collagen or poorly demarcated due to oedema and rarefaction. Unspecified gliosis was seen in close proximity to most cavities. Disrupted ependyma was found in all specimens. 'Pseudorosettes' were found within the syrinx space or in close relation to the syrinx wall. They were characterised by groups of ependymocytes surrounding one or more vessels, grey matter and collagen. Additionally, the study in question reported neuronal necrosis, Wallerian and spongy white matter degeneration and an impression of increased vascularity (Hu et al. 2012b).

Tissue slides from the same 12 dogs were used to investigate the semi-quantitative distribution of calcitonin gene-related peptide (CGRP) and SP-immunoreactivity in the C3 segment's grey matter (Hu et al. 2012a). As previously described, CGRP is primarily found in dorsal horn terminals of primary afferents and is involved in nociception. Symptomatic CKCS with an asymmetrical syrinx had a marked distortion of the dorsal horn SP- and CGRP-immunoreactive areas in the affected, compared to the non-affected, half of the spinal cord. In addition, a significant reduction in SP-immunoreactivity was seen when comparing pre-calculated ratios between immunopositive and -negative areas of the dorsal quadrants in symptomatic CKCS and controls (Hu et al. 2012a).

Treatment and prognosis

There are no available licensed compounds with the indication neuropathic pain for veterinary species. According to the American Animal Hospital Association and the American Association for Feline Practitioners' pain management guidelines, chronic pain may be managed by opioids, NSAIDs, gabapentin, amantadine and TCAs (Epstein et al. 2015). A number of suggestions have been made to manage the specific syringomyelia-associated clinical signs in CKCS: NSAIDs, corticosteroids, diuretics, proton pump inhibitors, opioids, gabapentinoids and amitriptyline (Rusbridge 2005; Rusbridge & Jeffery 2008; Plessas et al. 2012). None of these suggested compounds have proven sufficient effect in the reduction of clinical signs of neuropathic pain. Nevertheless, a single-blinded trial of gabapentin as an add-on to carprofen in CKCS with clinical signs of syringomyelia reported a significant improvement in quality of life as an indirect indicator of pain alleviation when compared to baseline (Plessas et al. 2015).

Forms of surgical management, including foramen magnum decompression and syringo-subarachnoid shunting, have all been trialled to prevent syrinx progression and to reduce clinical signs (Dewey et al. 2004; Vermeersch et al. 2004; Motta & Skerritt 2012). Post-operative outcome assessment varies between the studies. Overall, initial improvement in clinical status is seen in up to 80% of cases. However, in long-term follow-up reports, up to 50% of cases relapse after foramen magnum decompression (Dewey et al. 2004; Vermeersch et al. 2004; Rusbridge 2007). Most dogs continue the same medical treatment regimen as before surgery, and resolution of syringomyelia has not been reported (Dewey et al. 2004; Vermeersch et al. 2004; Rusbridge 2007).

To summarise, as in humans, syringomyelia-related neuropathic pain in the CKCS is fundamentally different from other types of pain. The pain phenotype is very diverse, and the pathogenesis is not fully understood (Driver et al. 2013). In addition, an insufficient response to treatment is seen in both human and canine patients. As a consequence, and due to the progressive nature of the disease, up to 15% of CKCS are euthanised due to an unacceptable quality of life (Plessas et al. 2012). The research project presented in this thesis was undertaken to provide novel information on neuropathic pain in CKCS with syringomyelia and to investigate if these dogs represent a spontaneous model of syringomyelia-related CNeP with translational potential.

Results - review and discussion of individual papers

The dynamic progress of the research project is presented in the following sections. First, the rationale behind each of the Studies I-IV is given, followed by a review of the main results and a discussion of these. For further details of the Studies I-IV the reader is referred to the four original Papers I-IV, pages 51-117. These findings and their relevance to the overall aim – that is, to investigate if the CKCS with syringomyelia represent a spontaneous model of CNeP - are discussed in the following section.

Study population

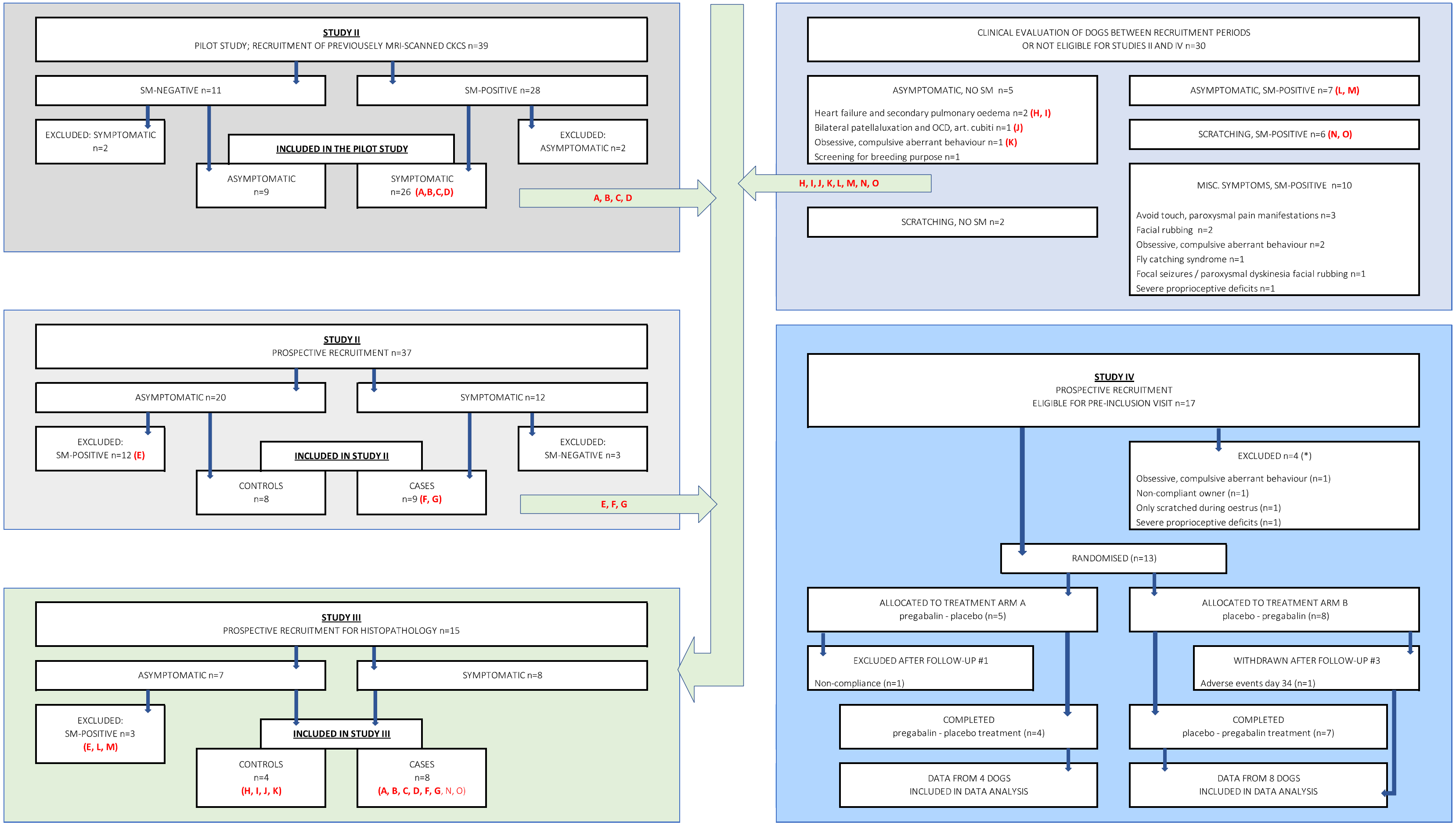
Dogs were recruited through referring colleagues in primary practices, from among cases in the neurology referral clinic at the University Hospital for Companion Animals in Copenhagen, and by contact with relevant breeding organisations and interest groups. Dogs who qualified for inclusion in Studies I-IV were purebred, client-owned Danish CKCS. Inclusion in any of the four studies was contingent upon written consent from each owner before undertaking any clinical work-up. A flow diagram of dogs included in Study I can be found in Paper I, page 55. The profile of Studies II-IV, including a detailed overview of the 119 dogs that underwent clinical characterisation between 2013 and 2018, is presented in Figure 4. In addition, blood samples for an ongoing genome-wide association study (GWAS) were collected from 116 dogs after clinical characterisation including MRI. The purpose of the ongoing GWAS is to investigate if one or more major genes are involved in the development of symptomatic syringomyelia in the CKCS. The study is outside the scope of this thesis and will therefore not be included in the present thesis.

Clinical classification of controls and cases

To be enrolled in Studies I-IV, controls should not express any clinical signs associated with syringomyelia, should not have a history of neurological or musculoskeletal conditions, or have been treated with analgesics or anti-epileptic medication less than six weeks prior to inclusion. The classification of a dog being symptomatic or asymptomatic was based on owner interview in Studies I-IV and the presence or absence of symptoms were confirmed by a clinical / neurological examination. This, however, excluded dogs included in the prevalence- and heritability study as described in Paper I. In general, symptomatic cases should as a minimum express uni- or bilateral scratching directed at the neck or shoulder area. The classification of a dog as being syringomyelia-positive or negative was based on MRI. The specific in- and exclusion criteria are described in detail in the respective original papers.

Figure 4 (next page)   Study profile, studies II-IV.

An overview of the 119 dogs that underwent clinical characterisation from 2013 - 2018. Red letters in parentheses demonstrate dogs that were donated for Study III. Blood samples were collected from 116 dogs after clinical characterisation including MRI for an ongoing genome-wide association study (GWAS). Three dogs that were excluded before enrollment in the clinical trial did not donate blood samples. These dogs are indicated with an asterix (*).


STUDY II
PILOT STUDY; RECRUITMENT OF PREVIOUSELY MRI-SCANNED CKCS n=39
SM-NEGATIVE n=11
SM-POSITIVE n=28
EXCLUDED: SYMPTOMATIC n=2
INCLUDED IN THE PILOT STUDY
EXCLUDED: ASYMPTOMATIC n=2
ASYMPTOMATIC n=9
SYMPTOMATIC n=26 (A,B,C,D)
A, B, C, D
STUDY II
PROSPECTIVE RECRUITMENT n=37
ASYMPTOMATIC n=20
SYMPTOMATIC n=12
EXCLUDED: SM-POSITIVE n=12 (E)
INCLUDED IN STUDY II
EXCLUDED: SM-NEGATIVE n=3
CONTROLS n=8
CASES n=9 (F, G)
E, F, G
STUDY III
PROSPECTIVE RECRUITMENT FOR HISTOPATHOLOGY n=15
ASYMPTOMATIC n=7
SYMPTOMATIC n=8
EXCLUDED: SM-POSITIVE n=3 (E, L, M)
INCLUDED IN STUDY III
CONTROLS n=4 (H, I, J, K)
CASES n=8 (A, B, C, D, F, G, N, O)
CLINICAL EVALUATION OF DOGS BETWEEN RECRUITMENT PERIODS
OR NOT ELIGIBLE FOR STUDIES II AND IV n=30
ASYMPTOMATIC, NO SM n=5
Heart failure and secondary pulmonary oedema n=2 (H, I)
Bilateral patellaluxation and OCD, art. cubiti n=1 (J)
Obsessive, compulsive aberrant behaviour n=1 (K)
Screening for breeding purpose n=1
ASYMPTOMATIC, SM-POSITIVE n=7 (L, M)
SCRATCHING, SM-POSITIVE n=6 (N, O)
H, I, J, K, L, M, N, O
SCRATCHING, NO SM n=2
MISC. SYMPTOMS, SM-POSITIVE n=10
Avoid touch, paroxysmal pain manifestations n=3
Facial rubbing n=2
Obsessive, compulsive aberrant behaviour n=2
Fly catching syndrome n=1
Focal seizures / paroxysmal dyskinesia facial rubbing n=1
Severe proprioceptive deficits n=1
STUDY IV
PROSPECTIVE RECRUITMENT
ELIGIBLE FOR PRE-INCLUSION VISIT n=17
EXCLUDED n=4 (*)
Obsessive, compulsive aberrant behaviour (n=1)
Non-compliant owner (n=1)
Only scratched during oestrus (n=1)
Severe proprioceptive deficits (n=1)
RANDOMISED (n=13)
ALLOCATED TO TREATMENT ARM A
pregabalin - placebo (n=5)
ALLOCATED TO TREATMENT ARM B
placebo - pregabalin (n=8)
EXCLUDED AFTER FOLLOW-UP #1
Non-compliance (n=1)
WITHDRAWN AFTER FOLLOW-UP #3
Adverse events day 34 (n=1)
COMPLETED
pregabalin - placebo treatment (n=4)
COMPLETED
placebo - pregabalin treatment (n=7)
DATA FROM 4 DOGS
INCLUDED IN DATA ANALYSIS
DATA FROM 8 DOGS
INCLUDED IN DATA ANALYSIS

Clinical characterisation

The characterisation of the Danish population of CKCS was initiated in 2007 by Professor Mette Berendt. All included dogs underwent a standardised clinical characterisation to ensure a consistent case classification within and between Studies I-IV. Dogs included in Study I were characterised by Professor Berendt, whereas the dogs included in Study II-IV were characterised by the present author, hereafter designated the principal investigator (PI). A standardised screening questionnaire was designed and used in Study I. The questions were based on previous reports outlining clinical signs associated with SM (Rusbridge et al. 2000; Rusbridge et al. 2006). The questionnaire published by Rutherford *et al*. (Rutherford et al. 2012) included questions regarding each dog's general health status, medical history, clinical signs, behaviour and quality of life. These questions were used to confirm eligibility and to establish each individual dog's baseline neuropathic pain score before inclusion in Studies II-IV.

After history uptake, a standardised clinical and neurological examination was undertaken. Blood samples were collected for haematology and a biochemical profile. The clinical characterisation of the dogs in Studies II-IV additionally included otoscopy and microscopy of cerumen, urinalyses after cystocentesis and a thyroid profile including T_4, fT_4 and TSH. Blood and urine samples were analysed at the Veterinary Diagnostic Laboratory, Copenhagen.

Neuroimaging

All dogs included in Studies I-IV underwent MRI (0.2 T Esaote Vet MR, Italy) under general anaesthesia. The MRI protocol included the following sequences: T1W transverse, sagittal and dorsal images and T2W transverse and sagittal images. Cases underwent MRI of the neurocranium and spinal cord segments C1-C6. Screening of controls included one region from the interthalamic adhesion and as far caudally as possible but at least to the C4/C5 intervertebral disc space (Cappello & Rusbridge 2007). For a detailed description of field of view, time to echo, time to repeat slice gap, slice thickness and number of excitations, please see Appendix A in Paper II, page 67. The MR images were assessed immediately after they were obtained by the PI. A subsequent masking of the MRI series was undertaken, and a unique study number was assigned before transfer to the web-based DICOM image viewing system RemoteEye (NeoLogica, Italy). This enabled blinded, standardised description by a Resident (Studies I and II) or a Diplomate of the European College of Veterinary Diagnostic Imaging (Study IV).

The prevalence and heritability of symptomatic syringomyelia - Study I

The overall purpose of Study I was to characterise the Danish CKCS population by estimating the prevalence and heritability of symptomatic syringomyelia, by investigating the association between clinical signs and MRI findings and by reporting the long-term outcomes in symptomatic and asymptomatic dogs.

A cross-sectional study was undertaken to estimate the prevalence of symptomatic syringomyelia in the Danish population. The study population consisted of all CKCS (n=240) born and registered in the Danish Kennel Club in 2001. Consequently, the dogs were all six years of age when the study was undertaken. Screening questionnaires addressing clinical signs of syringomyelia were mailed to the 240 owners. One-hundred and twenty-three responders participated. Nineteen symptomatic dogs were identified after telephone interview validation. The classification of symptomatic dogs was confirmed in 14/19 (74%) of the dogs after clinical examination. The estimated prevalence of symptomatic syringomyelia in six-year-old Danish CKCS born in 2001 was 15.4% (CI_{95} = 9%, 22%).

The estimation of heritability for symptomatic syringomyelia was based on the disease frequency in the general population and among relatives. To investigate the prevalence of disease in families, eight dogs were selected among the symptomatic individuals identified in the prevalence study. Information on litter mates was collected for all full siblings to the eight symptomatic dogs. The eight litters comprised a total of 34 siblings, with 17 asymptomatic and 17 symptomatic dogs. The incidence of symptomatic and asymptomatic dogs was converted to normal distributed mean liabilities, and their standard deviation from the threshold was used to estimate the heritability of symptomatic syringomyelia in dogs older than six years of age. A high heritability of 0.81 was found. In support of the high heritability, there was a significant difference ($\chi^2 = 9.3$; df = 1; $P<0.05$) between the prevalence of symptomatic syringomyelia in the population (15.4%) and in the full siblings related to the eight symptomatic dogs identified in the prevalence study. Despite the polygenically determined genotype with variable penetrance (Lewis et al. 2010) this finding indicates that genetics have a strong impact on the total phenotypic variance in the population.

After the publication of Paper I, a prevalence study was undertaken in CKCS of all ages attending primary care in England over a five-year period. It demonstrated, that 65/4046 (1.6%) CKCS revealed clinical signs suggestive of CM-SM (Sanchis-Mora et al. 2016). The data was obtained by screening a database of clinical information (case notes and prespecified diagnosis codes) entered by an unspecified number of veterinarians, and the case-definition was accordingly dissimilar to the stricter case-definition used in Study I. The demand of MRI for a definitive diagnosis of syringomyelia affects the prevalence estimates of syringomyelia in the general population. In addition, the presence or absence of a syrinx on MRI is not interchangeable with this finding being clinical relevant. The prevalence of asymptomatic syringomyelia was estimated to 46% in a retrospective assessment of 555 screening MRIs for breeding purpose in CKCS from the United Kingdom and the Netherlands (Parker et al. 2011). The prevalence may be affected by selection bias due to the purpose of the MRI, and the clinical status was based on owner-assessment, not confirmed through clinical examination.

The association between clinical findings and MRI - Study I

The hypothesised association between the expression of symptoms and MRI-findings was investigated in the 34 littermates identified in the prevalence- and heritability study. Twenty-two dogs underwent clinical characterisation including MRI. Clinically, 13 dogs were symptomatic and nine were asymptomatic. On MRI, syringomyelia was found in 13/13 (100%) symptomatic dogs and in 8/9 (89%) asymptomatic dogs. The presence of syringomyelia was not significantly associated with the expression of clinical signs ($P=0.41$). The positive predictive value of MRI, i.e. the probability that a dog was symptomatic if syringomyelia was present, was 0.62.

The diameter of the syrinx and the syrinx / spinal cord ratio was significantly different between symptomatic and asymptomatic dogs. The hypothesised association between the expression of symptoms and (1) syrinx diameter and (2) syrinx / spinal cord ratio was confirmed (P<0.01 for both comparisons).

The frequent occurrence of syringomyelia in middle-aged, asymptomatic dogs reported in Study I was consistent with previous and later reports. The occurrence of asymptomatic syringomyelia increases considerably with age from 25% in dogs up to 12 months of age up to 70% in dogs ≥ six years of age (Parker et al. 2011; Cerda-Gonzalez et al. 2016). The lack of association between the presence of a syrinx on MRI and expression of clinical signs consistent with syringomyelia reported in Study I was confirmed by Sparks *et al.* (Sparks et al. 2018a).

In addition, previously reported findings of syrinx width as a strong predictor of symptoms was confirmed in Study I (Rusbridge et al. 2007). However, Sparks *et al*. (2018a) found no difference between symptomatic and asymptomatic dogs when comparing the maximum syrinx height expressed as a percentage of the spinal cord height. The difference in findings between the studies likely reflects two different approaches to the MRI measurements. Sparks *et al*. measured the maximum height of the syrinx at a fixed, dorso-ventral position. In comparison, the syrinx/spinal cord ratio reported in Study I was measured where the syrinx was widest since symptomatic syringomyelia is often asymmetric and lateralised, primarily affecting one dorso-lateral quadrant of the spinal cord (Rusbridge et al. 2007; Hu et al. 2012b).

Long-term follow-up of clinical status - Study I

To elucidate whether the clinical phenotype was changing over time, a five-year follow-up study was undertaken in 2012. Information on 31/34 littermates (16 symptomatic and 15 asymptomatic) identified in the prevalence and heritability study was available for follow-up. Based on the owners' assessments, 14/15 (93%) of asymptomatic dogs remained asymptomatic while 13/16 (81%) of symptomatic dogs remained so. Progression of symptoms was seen in 4/13 (31%) symptomatic dogs. The symptoms ceased in 3/16 previously symptomatic dogs; in one dog this was due to sufficient treatment. Overall, 20/31 (65%) dogs had been euthanised; this included 4/16 (25%) symptomatic dogs due to insufficient alleviation of their symptoms and an unacceptable quality of life despite treatment.

Regarding the 22 littermates that underwent clinical characterisation including MRI in 2007, follow-up information could be obtained on 20/22 dogs. Eleven of these dogs were identified as symptomatic and syringomyelia-positive in 2007. In that year, eight of the nine asymptomatic dogs were also syringomyelia-positive. The owners reported that 10/11 (91%) symptomatic, syringomyelia-affected dogs remained symptomatic; meanwhile, 8/9 (89%) asymptomatic dogs had remained asymptomatic. Only one asymptomatic, syringomyelia-affected dog developed symptoms of syringomyelia during the five-year period.

The owner-assessed static clinical phenotype was later confirmed in 54 CKCS (16 symptomatic and 38 asymptomatic) by Cerda-Gonzales *et al.* The majority (68%) of asymptomatic dogs remained asymptomatic, and most (88%) symptomatic dogs remained symptomatic; progression of clinical signs was seen in 56% of symptomatic dogs < 36 months after MRI. The symptoms improved with medical treatment in 2/16 (13%) symptomatic dogs. Three of these 54 CKCS (6%) had been euthanised due to myelopathy or signs of clinical deterioration and worsened pain (Cerda-Gonzalez et al. 2016). In a follow-up study in 79 owner-assessed asymptomatic CKCS (54 without syringomyelia and 25 with syringomyelia), 4/54 (7%) syringomyelia-negative dogs developed symptoms 2.6 years after MRI. The clinical status changed from asymptomatic to symptomatic in 9/25 (36%) syringomyelia-affected dogs (Ives et al. 2015). A relatively high euthanasia-rate has also been reported in 7/48 (15%) of CM-affected, symptomatic CKCS whereof 39 had concomitant syringomyelia (Plessas et al. 2012).

In conclusion, Study I demonstrated that the prevalence of symptomatic syringomyelia is high (15.4%) in the Danish population of middle-aged CKCS. The heritability is very high (0.82), and the prevalence of symptomatic syringomyelia is significantly higher in litters where at least one full sibling is symptomatic, compared to the general population. This finding indicates, that genetics have a strong impact on the total phenotypic variance in the population. The positive predictive value of MRI is moderate (0.62), hence the presence of a syrinx on MRI does not imply that the dog is symptomatic. However, there is a significant association between the expression of symptoms and specific MRI findings; symptomatic dogs have larger syrinx diameter and larger syrinx/spinal cord ratio compared to asymptomatic dogs. Moreover, the clinical status of middle-aged CKCS is static. When symptomatic syringomyelia has developed, it persists and may progress over time. Spontaneous recovery is less likely. The likelihood that older, asymptomatic syringomyelia-positive dogs develop symptoms is low. Treatment of symptomatic dogs is difficult and the euthanasia-rate is high due to ineffective treatment regimens and progression of symptoms over time.

Mechanical sensory threshold quantification - Study II

With a case definition based on a history of pain, a confirmation of the symptoms on clinical examination and a further confirmation of a nervous system lesion on MRI, the next rational step was to elucidate whether a sensory assessment might confirm the presence or absence of specific sensory abnormalities. Tactile allodynia and mechanical hyperalgesia have been reported as frequently occurring sensory abnormalities in human syringomyelia-patients with CNeP (Hatem et al. 2010). The clinical characteristics of symptomatic, syringomyelia-affected CKCS have been associated with signs of allodynia and hyperalgesia (Rusbridge et al. 2007; Rusbridge & Jeffery 2008; Hu et al. 2012b; Plessas et al. 2012; Schmidt et al. 2013).

To investigate if the clinical phenotype and behavioural indicators of pain were associated with mechanical sensory threshold (MST) alterations, a case-control study was initiated towards the end of 2013. It was hypothesised that syringomyelia would result in a significant difference in the MST between symptomatic, syringomyelia-positive CKCS and asymptomatic, syringomyelia-negative CKCS.

Initially, a methodological pilot study was undertaken in previously MRI-scanned CKCS. Nine non-affected, asymptomatic CKCS and 26 affected, symptomatic CKCS were included. The primary aim was to assess the dogs' reaction to cutaneous stimulation with monofilaments (Touch Test® Sensory Evaluators). The MST quantifications were video-recorded. The videos were subsequently reviewed to establish a catalogue of reactions (an ethogram) elicited by this form of stimulation. The nine most prevalent behavioural reactions elicited by monofilament stimulation were:

- Eye twitching, blinking, lifting the eyelids with lateral movement of the eyes directed at the stimulation side without turning the head.
- Subtle attentional shift characterised by a physical orientation directed at the stimulation side; the head is turned toward the stimulus, but the lateroflexion of the neck is < 90° from the midline.
- Ear twitch.
- Stops panting ≥ two seconds.
- Distinct attentional shift characterised by a physical orientation directed at the stimulation side; the head is turned toward the stimulus, but the lateroflexion of the neck is > 90° from the midline.
- Body twitch.
- Spontaneous headshake.
- Evoked scratching.
- Avoidance / withdrawal response characterised by a distinct movement away from the stimulus.

A mechanical sensory threshold quantification (MSTQ) study was designed based on the results from the pilot study. A prospective case-control was undertaken, and nine symptomatic, syringomyelia-affected CKCS and eight asymptomatic CKCS without syringomyelia were included. The primary outcome was defined as the monofilament size in grams that would elicit one of the five behavioural reactions: distinct attentional shift; body twitch; spontaneous headshake; evoked scratching or avoidance / withdrawal response. The response should be reproducible, meaning that the same response should be elicited by two consecutive stimuli of the same intensity. The MST was quantified on both sides of each dog's neck, and the individual dog's final MST was reported as a mean of the paired measurements.

The MST range varied considerable within and between cases and controls. The unpaired comparison of the log10-transformed mean MST between cases and controls was insignificant (P=0.25). In addition, no significant differences were found between cases and control when comparing the initial MST with the MST on the contralateral side of the neck. Comparisons of the mean MST between dogs with symmetric and asymmetric syringes and between the affected and non-affected side in cases with unilateral scratching were likewise insignificant.

The hypothesised difference in MST in syringomyelia-affected, symptomatic CKCS compared to non-affected, asymptomatic CKCS could not be confirmed.

The inconclusive findings may be caused by an underpowered study design due to the small sample size and large MST variation within and between dogs.

Prior to the initiation of Study II, the use of monofilaments had been reported in one study to assess the sensory threshold in dogs with a naturally occurring painful condition (Brydges et al. 2012). A comparison of MST was undertaken within and between 11 dogs with unilateral cranial cruciate ligament rupture and 15 healthy controls. The sensory threshold was lower in the limb affected by cranial cruciate ligament rupture when compared to the contralateral limb. Later, a prospective study in 44 CKCS (14 without syringomyelia and 30 with syringomyelia) quantified the mechanical threshold by application of a haemostatic forceps with an attached digital load cell to the lateral digits of the thoracic limbs and on both sides of the neck (Sparks et al. 2018b). The threshold was defined by vocalisation or escape behaviour. The study was unable to demonstrate a relationship between the presence or severity of syringomyelia and the quantified mechanical threshold. Pain was present in 30/44 dogs on neurological examination. The 30 dogs in pain, of which an unknown number received pain medication on the day for testing, had lower MSTs on both paw and neck compared to dogs not in pain.

In 2017, a sensory threshold examination protocol (STEP) was developed and an initial validation was undertaken in 25 healthy dogs of different sizes and ages to enable subsequent phenotyping of canine pain syndromes (Sanchis-Mora et al. 2017). The tactile and mechanical thresholds were quantified with von Frey's filaments and a pressure algometer respectively. Thresholds were quantified on the tibia, humerus, neck, thoraco-lumbar area and the abdomen on both sides of the body. The endpoints were defined as one of four behavioural responses: turning the head towards the device, growling, lip-licking or backing away from the stimulus. Factors that affect the MSTQ in dogs were: body area tested, the age and the size of the dog. Higher thresholds were quantified on the neck when compared to the thresholds of other body areas, young dogs (0.3-3 years of age) had higher thresholds when compared to adult dogs (4-6 years of age) and female dogs had higher thresholds when compared to male dogs. Small dogs weighing 1-8 kg had lower mechanical thresholds when compared to medium dogs (9-22 kg) and large dogs (23-40 kg). Lower thresholds were also found in geriatric dogs (> 6 years of age) when compared to adult dogs.

The initial validation of the STEP-protocol reflects the overall challenge of mechanical sensory testing in healthy dogs. The MST quantification (MSTQ) outcome varies tremendously within and between dogs and is affected by several factors including age, sex, body size and the body area tested. In addition, the behavioural response elicited by stimulation with the monofilament is determined by the PI's subjective assessment. This was acknowledged during the development of the ethogram used in Study II. Meticulous, repeated evaluation of the 35 videos of MSTQ in previously MRI-scanned dogs revealed both subtle and distinct behavioural responses. In human neuropathic pain patients, monofilaments are used to assess the mechanical pain (Aδ-fibre) threshold. Whether the healthy dogs' behavioural responses to monofilament stimulation is a reaction to light (Aβ) or Aδ- activation is simply not possible to assess. Consequently, it is even more challenging to assess and interpret responses elicited in dogs with a naturally occurring painful condition and anticipated altered thresholds. Hence, the case definition of neuropathic pain in dogs must for now remain tentative based on a history of pain and a confirmatory clinical examination including MRI.

Histomorphology: quantification and characterisation of spinal cord lesions - **Study III**

There are only sparse descriptions of histomorphological characteristics and their association with CNeP in dogs as well as humans with Chiari-malformation and syringomyelia. In addition, the proposed biochemical and functional consequences of the central nervous system lesions are poorly understood as are their relationship to the symptoms of CNeP. For these reasons, a design-based stereological quantification and histopathological characterisation of spinal cord lesions in syringomyelia-affected, symptomatic CKCS was begun towards the end of 2017.

The purpose of this study was to elucidate whether one or more specific structural cervical spinal cord entities involved in nociception were affected by syringomyelia in symptomatic dogs. It was hypothesised, that a relationship between unilateral symptoms of CNeP and quantifiable structural loss of a specific anatomical cervical spinal cord entity in CKCS with syringomyelia could be established.

The spinal cord segments C1-C8 from eight symptomatic, syringomyelia-affected CKCS and four asymptomatic, nonaffected CKCS were investigated. Seven of the eight cases had previously been enrolled in Study II (Figure 4). The dogs' symptoms of CNeP had previously been well-controlled with different treatment regimens. Due to disease progression and aggravation of symptoms, it was no longer possible to alleviate their symptoms and sustain an acceptable quality of life. Euthanasia was therefore decided for ethical reasons. Controls were included if they were to be euthanised for any reason other than symptomatic syringomyelia. All dogs had undergone the standardised clinical characterisation including MRI, as previously noted, except for three of the four asymptomatic controls, where MRI was not indicated.

Tissue-sections were immuno-stained with SMI-32 (Biolegend, Denmark), a primary monoclonal mouse-antibody previously shown to label neurofilament triplet H proteins in rat, human and mouse nervous tissue (Ma 2001; Petzold et al. 2011; Turner et al. 2015). As demonstrated in Figure 5, the immune-labelling enabled visualisation of the boundaries between the dorsal horn's laminae III and IV (Watson et al. 2008).

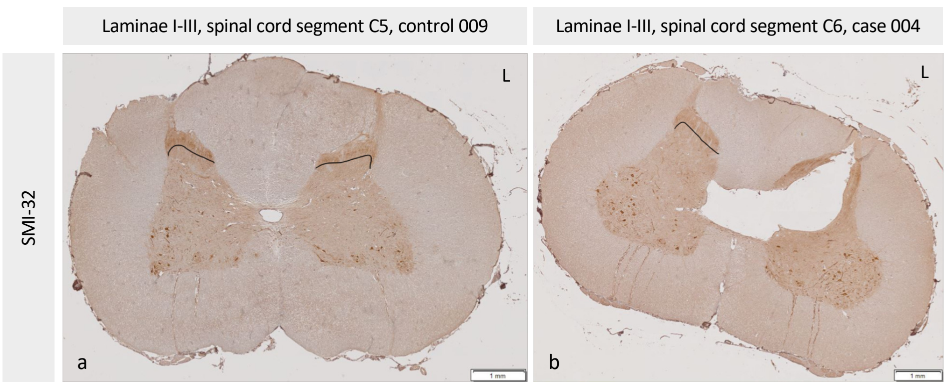


Figure 5 Immuno-labelling of dorsal horn laminae I-III.
Photomicrographs of 10 µm thick transverse sections. The micrographs demonstrate examples of SMI-32 immunoreactivity in the spinal cord dorsal horn's laminae I-III in **a)** a 2.5-year-old female control and **b)** a 4.9-year-old male case with unilateral scratching on the left side of the neck. **L**, left side of the spinal cord.

Stereological volume quantification

Stereology is a tool used to estimate three-dimensional information about a tissue or organ based on two-dimensional quantification in tissue sections (Gundersen et al. 1988). This design-based quantification relies on statistics and stochastic geometry and uses well-defined sampling techniques. In Study III, systematic uniformly random sampling was used. Here, the sampling of tissue blocks starts at random, and the subsequent tissue blocks are sampled at regular, predetermined intervals. In addition, the method requires that the selected tissue blocks represent the entire organ of interest (Boyce et al. 2010). A geometrical probe is used to collect information or 'events', as described below (Evans & Nyenaard 2007). In Study III, this method was used to estimate the total volume of the spinal cord segments C1-C8 and relevant sub-volumes of *inter alia* the specific structural cervical spinal cord entities involved in nociception as described in Paper III.

Overall, unpaired comparisons of the total mean volumes and sub-volumes revealed no significant difference between cases and controls. Seven of the eight included symptomatic, syringomyelia-affected dogs expressed unilateral symptoms of CNeP. Paired comparisons of the estimated sub-volumes were undertaken between the affected and non-affected (left and right) halves of these seven dogs' spinal cords. A significant volume loss of the dorsal horn laminae I-III was found on the affected side, the side where symptoms had been confirmed during the dog's lifetime (P=0.034).

Hence, the hypothesised relationship between unilateral symptoms of CNeP and a quantifiable structural loss of a specific anatomical cervical spinal cord entity in CKCS with syringomyelia was confirmed.

This is the first report identifying a quantified loss of dorsal horn grey matter. Although there have been previous reports on grey matter loss primarily affecting the dorsal horn in human and canine syringomyelia-patients, these were subjectively assessed as opposed to clearly quantified (Hinokuma et al. 1992; Hu et al. 2012b). On the other hand, additional comparisons of the total volumes of grey matter laminae IV-X, dorsal, lateral and ventral columns were insignificant. Moreover, the total volumes of the spinal cord and sub-volumes were not significant different between cases and controls. It is important to note that the estimated volumes are subject to rather large tissue shrinkage due to paraffin embedding (Dorph-Petersen et al. 2001).

Descriptive histomorphology

A systematic morphological assessment of all tissue slides from spinal cord segments C1-C8 was undertaken as described in Paper III. Contrary to the previously evinced association between unilateral symptoms and an asymmetric syrinx distribution on MRI (Rusbridge et al. 2007; Thofner et al. 2015), the left/right asymmetry was very inconsistent within the individual spinal cords. In 6/7 cases, the asymmetry alternated between the symptomatic and asymptomatic halves of the spinal cord. However, another consistent pattern was seen: in 7/7 cases with unilateral clinical signs, the ipsilateral dorsal root entry zone (DREZ) had an abnormal appearance. The DREZ was degenerated, and disruption of the pia mater was seen in one or more segments, most frequently in C3 (4/7 cases) and C4 (3/7 cases). In addition to the loss of superficial dorsal horn grey matter, a concomitant axonal loss was seen. Moreover, there was evidence of a reorganisation of first-order neurons characterised by termination in deeper laminae of the grey matter when compared to contralateral dorsal horns. These changes have not previously been described in CKCS with syringomyelia-related CNeP. Degeneration of the DREZ with or without communication to the subarachnoid space has been described in nine human cases (Netsky 1953; Hinokuma et al. 1992; Milhorat et al. 1995; Beuls et al. 1996). Localisation of the patients' symptoms were noted in 5/9 cases. These five cases presented unilateral paraesthesia, hypo- or hyperaesthesia and pain. The sensory disturbances were localised in the relevant side and dermatomal areas corresponding to the spinal cord segments affected by DREZ degeneration (Netsky 1953; Milhorat et al. 1995; Beuls et al. 1996).

In Study III, the predominant symptom of CNeP was scratching in 6/7 dogs with unilateral symptoms. Unilateral pain on cervical palpation was the prominent feature in 1/7 dogs. Ectoparasites, dermatitis and other dermal conditions that usually lead to scratching in dogs had been ruled-out during the clinical examination prior to euthanasia. Here it should be noted that, unlike the well-characterised nociceptive pathway, we have not yet established a corresponding itch-specific pathway (Koch et al. 2018). Accordingly, the underlying functional and biochemical mechanisms of scratch in CKCS with syringomyelia-related CNeP remains unknown so far. Under normal conditions, itch is transmitted via pruritoceptive fibres. These fibres synapse with excitatory neurons in the dorsal horns' laminae I-IV (Koch et al. 2018). The nociceptive Aδ- and C-fibres synapse in laminae I-II, whereas innocuous Aβ-input is primarily received in laminae III and IV (Benarroch 2016). In an experimental rodent model of excitotoxic spinal cord injury, Yeziersky *et al.* reported behavioural indication of neuropathic pain characterised by excessive grooming and lowered mechanical thresholds despite sparring of superficial dorsal horn grey matter (Yezierski et al. 1998). Study III could not confirm whether the DREZ-pathology in the affected, symptomatic CKCS serves as a functional explanation of the experimental evidence of neuropathic itch and mechanical threshold alterations as described by Yeziersky *et al*. (Yezierski et al. 1998). Although the MST had been quantified on both sides of the neck in all included cases, an anatomical mapping of the thresholds and assessment of potential threshold alteration was not undertaken.

The somatosensory function has been evaluated in eight human patients with syringomyelia-associated CNeP by application of laser-evoked potentials (Kakigi et al. 1991). In 7/8 patients, the function of the ascending fibres was intact, whereas an impaired dorsal horn function was reported in 6/8 patients. Ducreux *et al*. have shown that spinothalamic pathway lesions alone cannot explain the development of CNeP in syringomyelia-patients (Ducreux et al. 2006). In 31 patients with CNeP, 11/31 presented spontaneous ongoing pain, whereas 20/31 presented allodynia. Moreover, the pattern of sensory deficits was different in the two groups: Patients with spontaneous ongoing pain had more severe thermal and tactile sensory deficits compared to patients with allodynia. According to the authors, this suggests that the neuropathic pain in this group of patients was related to deafferentation.

In conclusion, an association between lateralised symptoms and the structural grey matter volume loss was established in Study III. Moreover, a degeneration of the DREZ with dissection through pia mater was found in dogs with lateralised symptoms. These findings offer a structural explanation of the CNeP symptoms in CKCS with syringomyelia. However, whether the symptoms are a consequence of primary deafferentation with trans-synaptic degeneration of the dorsal horn or a primary loss of dorsal horn neurons with secondary deafferentation remains unknown so far.

Efficacy of pregabalin, a randomised controlled trial - Study IV

It is a common understanding within the veterinary profession that the clinical characteristics of symptomatic, syringomyelia-affected CKCS are indicators of pain (Rusbridge et al. 2006; Rusbridge & Jeffery 2008; Rutherford et al. 2012; Sanchis-Mora et al. 2016; Cockburn et al. 2018; Hechler & Moore 2018). The euthanasia-rate of symptomatic CKCS with syringomyelia is high due to an insufficient response to treatment. Furthermore, there is a lack of licensed compounds and evidence-based treatment protocols. Pregabalin (PGN) is a first-line analgesic for treatment of human neuropathic pain patients.

Study IV, a randomised, double-blind, placebo-controlled cross-over trial was conducted in 2017-2018. The aim was to assess if PGN could function as an effective treatment of syringomyelia-related pain in CKCS and thereby to investigate the model's translational potential. It was hypothesised that the analgesic efficacy of PGN is superior to placebo to reduce neuropathic pain symptoms in CKCS with syringomyelia.

Thirteen symptomatic, syringomyelia-affected dogs were included in Study IV after standardised clinical characterisation. The dogs were randomised to treatment arm A (PGN → placebo; n=5) or treatment arm B (placebo → PGN; n=8). The two treatment periods were of a duration of 25 days, segregated by a 48-hour wash-out period before cross-over. The primary outcome was the number of scratching events during ten minutes of exercise. Standardised video-series lasting ten minutes were obtained to quantify and document the number of scratching events at baseline and at four follow-ups. Secondary outcome measures were the owner's and the PI's assessment of each of the dog's scratching intensity and degree of pain / discomfort on the 11-point NRS-scale and on a modified Pain Faces Scale with a corresponding VAS-scale. In addition, the owner and the PI rated the dogs' quality of life as 'could not be better', 'good', 'fairly good', 'neither good nor bad', 'fairly poor', 'poor', 'could not be worse' or 'do not know' at baseline and the four follow-ups.

The treatment effect was estimated using a generalised estimating equation (GEE). The GEE is a statistical regression tool used for analysing longitudinal data with repeated observations of an outcome which has a distribution other than the normal, Gaussian distribution. The GEE was applied to account for the correlation of scratching events over time observed in one particular dog. Since the number of scratching events is a count variable, it would be natural to use a Poisson distribution; yet, to account for a possible overdispersion, a Negative Binomial distribution was used instead. The effect of the covariates PGN or placebo, treatment period, follow-up visit number and potential carryover effect on the mean number of scratching events was modelled using a log-link so that all effects are assumed to be multiplicative.

Eleven dogs completed the trial. One dog was excluded after follow-up visit 1 due to non-compliance. One dog was withdrawn by the owner after follow-up visit 3. After cross-over from placebo to PGN the dog developed ataxia and somnolence. Since the GEE model accounts for values missing completely at random, data from 12 dogs (the eleven dogs that completed the trial and the one dog withdrawn after follow-up 3) were included in the analysis. The treatment effect of PGN on the mean number of scratching events was estimated to be a factor 0.16 (CI_{95} = 0.11, 0.25). The factor 0.16 corresponds to an 84% reduction from baseline in the mean number of scratching events during ten minutes of exercise when compared to placebo (P<0.0001).

Hence, the analgesic efficacy of PGN was found superior to placebo to reduce neuropathic pain symptoms in CKCS with syringomyelia.

Efficacy estimates of PGN on secondary outcomes showed a significant reduction from baseline in owner-reported mean scratching intensities on the NRS- (P=0.003) and VAS-scales (P<0.0001) and in mean pain intensities assessed on the VAS-scale (P=0.01). The same significant effect of PGN on PI-assessed secondary outcomes was seen on scratching intensities assessed on the NRS- (P=0.0016) and VAS-scale (P=0.001), and on pain intensities likewise assessed on the NRS- (P<0.0001) and VAS-scale (P<0.0001). The only exception, where no difference was seen in the effect between PGN and placebo, was on owner-assessed pain on the NRS-scale (P=0.056). The most prevalent side effects of PGN reported by the owners were increased appetite in 9/12 dogs and transient ataxia which resolved within one to ten days in 9/12 dogs. During treatment with PGN, the owner-assessed quality of life improved in 38% of dogs allocated to arm A (PGN → placebo) and in 36% of the cases allocated to arm B (placebo → PGN) relative to baseline assessment. None of the owners of dogs allocated to arm B reported improved quality of life during placebo treatment. Contrary, 25% of owners reported a quality-of-life improvement at follow-up 3, the first follow-up after cross-over from PGN to placebo. Due to the small sample size, it was not possible to determine whether this finding is significant.

Study IV is the first randomised, double-blind, placebo-controlled cross-over trial undertaken to investigate an effective treatment of syringomyelia-related pain in CKCS. Although not direct comparable, a single-blinded trial of gabapentin as add-on to the NSAID carprofen has been investigated in CKCS with symptomatic syringomyelia (Plessas et al. 2015). The study reported a significant improvement in quality of life assessed on a VAS-scale compared to baseline as an indirect indicator of pain alleviation.

The primary outcome in Study IV, the number of scratching events during ten minutes of exercise, was chosen as a measurable, indirect biomarker of pain. Despite the laborious assessment of video-recordings, the outcome measure was easily quantified. In addition, the videos were used for a more detailed mapping of the individual dog's scratching pattern, both with regard to the presence or absence of 'phantom scratching' and the anatomical area to which the scratching was directed. The dogs' scratching profile changed during treatment with PGN. At baseline, eight dogs had one or more 'phantom scratch' episodes during the ten minutes of outcome assessment. The phantom scratch stopped in 7/8 dogs during treatment with PGN. In addition to the scratching as a physical indicator of discomfort, the dogs' behavioural indicators of pain in terms of vocalisation when scratching ceased in five individuals (100%) during treatment with PGN compared to baseline. These behavioral indicators of pain are quantifiable outcome measures that can be used to assess treatment effect. Contrary, the NRS and VAS scales are not validated tools to assess scratching and pain intensities in dogs. The owner- and PI-assessment on these predefined scales are applicable in the assessment of each individual dog, but cannot at present be used to compare outcome between dogs enrolled in clinical trials.

The results from Study IV demonstrate that PGN functions as an effective treatment of syringomyelia-related pain in CKCS. This novel finding, in addition to that generated by Studies I-III, will be discussed in the following sections to elucidate whether the CKCS with syringomyelia represents a spontaneous model of CNeP with translational potential.

General discussion – a novel spontaneous model of neuropathic pain

The research presented in this thesis was undertaken to contribute to the current knowledge about neuropathic pain in CKCS with syringomyelia and investigate the potential of the dog as a spontaneous model of CNeP. The main findings of this thesis were that symptomatic syringomyelia has a high prevalence of 15% and a high heritability of 0.81 in CKCS. A number of physical and behavioural indicators of hypersensitivity, discomfort and pain are quantifiable common clinical features shared by the dogs. Among these are spontaneous scratching and evoked scratching, for example when touched, and vocalisation when scratching. The predictive value of MRI is moderate. However, an association between the expression of symptoms and (1) syrinx diameter and (2) syrinx/spinal cord ratio has been confirmed. Moreover, the clinical status of middle-aged CKCS is static. It could not be confirmed that the clinical phenotype of symptomatic syringomyelia is characterised by mechanical threshold alterations. The study on histomorphology has demonstrated a significant loss of dorsal horn grey matter and DREZ degeneration associated with clinical signs. Finally, PGN has been found effective in the reduction of clinical signs of neuropathic pain in the dogs. Altogether, these findings demonstrate several comparative aspects to human symptomatic syringomyelia. However, the functional, biochemical and structural causes of neuropathic pain in human and canine syringomyelia remain inadequately understood. Despite extensive preclinical research, there is a lack of experimental animal models that mimic the clinically relevant situation and the complexity of the human and canine neuropathic phenotype (Rice et al. 2018; Yezierski & Hansson 2018).

Phenotype classification

The applicability of the CKCS with MRI-confirmed syringomyelia as a good research model of CNeP relies on a valid and well-defined phenotype classification. However, as discussed in the present thesis as well as elsewhere, the clinical phenotype of the symptomatic CKCS is – at first glance – heterogeneous (Rusbridge & Jeffery 2008; Hechler & Moore 2018). The clinical case definition varies between studies with regard to clinical and behavioural characteristics regarded as consistent with syringomyelia. Hence, direct comparison between studies is difficult. In addition, the presence or absence of pain may or may not be included as a separate entity within the clinical phenotype characterisation. Some studies include cases with CM alone without concomitant syringomyelia, and the time from MRI to inclusion is also variable. Moreover, inclusion of syringomyelia-positive dogs irrespective of the presence or absence of clinical signs of CNeP adds to the inability to make comparisons between studies. Further, the clinical phenotype of symptomatic CKCS comprises of at least two comorbidities. The presence of CM in 99% of dogs and otitis media with effusion in up to 50% of dogs (Owen et al. 2004; Hayes et al. 2010; McGuinness et al. 2013; Ives et al. 2015). These two comorbidities are confounders, introducing uncertainty about the actual explanation of the symptoms, especially facial rubbing and scratching directed toward the ears, sleep disturbances and the aberrant behaviour designated ‘fly catching’ (Rusbridge & Jeffery 2008). In comparison, sleep disturbances are also a feature of the human C1M phenotype as are different types of headaches, visual and auditory disturbances (Milhorat et al. 1999). Nevertheless, it remains uncertain whether CM and otitis media with effusion contribute to the overall clinical phenotype in symptomatic CKCS. A definitive diagnosis of CNeP in dogs cannot be confirmed due to the inherent lack of verbal communication. However, the author of this thesis advocates that a strong tentative diagnosis is possible in symptomatic dogs based on a careful and thorough history uptake and clinical examination including MRI.

History uptake and clinical examination

Two non-validated assessment tools for dogs have been published since Study I was undertaken. In 2018, the ChiMPS-T questionnaire was presented as a clinical screening- and assessment tool addressing the medical history, frequency and severity of symptoms (Sparks et al. 2018a). The assessment of the symptomatic dogs includes a pain- and scratch map to outline the dog's affected body areas. This questionnaire was unable to establish a relationship between the presence of pain or scratching and syringomyelia in the 30 syringomyelia-affected dogs included in the study. In addition, there was a lack of correlation between the presence of pain determined after neurological examination and the owner-reported presence of pain, pain score and affected body area as indicated on the scratch- and pain-map. The reported findings of this study show that the tentative diagnosis of symptomatic syringomyelia cannot be based on owner-reported clinical signs alone. Moreover, the physical indicators of discomfort and pain and scratching are not concomitant findings in all symptomatic dogs.

The screening questionnaire used in Studies II-IV addresses the general health and medical status, behaviour and clinical manifestations of syringomyelia. When first published, the questionnaire was used to investigate the impact of clinical signs of neuropathic pain in the CKCS on the affected dogs' behaviour and quality of life (Rutherford et al. 2012). The study in question included 122 MRI-confirmed, symptomatic CKCS with CM-SM. Of these, 84 (69%) received treatment. In 9/120 (8%) answers, owners assessed their dogs' quality of life to be 'fairly poor'. The study found a significant positive correlation between the presence of neuropathic pain and behavioural changes including stranger-directed fear, non-social fear, separation-related behaviour and attachment behaviour. In turn, these forms of behaviours had previously been evaluated in 203 dogs with known behavioural anomalies compared with 1851 controls. The presence or absence of these aberrant behaviours were found valid to distinguish between dogs with and without behavioural problems (Hsu & Serpell 2003).

Based on these findings, the author of this thesis proposes an *extended symptom profiling* of symptomatic, syringomyelia-positive dogs. The standardised clinical characterisation used in Papers I-IV ensured a consistent case classification between the studies. It was imperative to include representative cases and controls only. Therefore, much emphasis was laid on ruling out other causes of scratching and pain. A future clinical phenotype characterisation would become much more specific by merging and refining the assessment tools published by Rutherford *et al.* (2012) and Sparks *et al.* (2018a) in combination with the novel information on the clinical phenotype provided in Studies I-IV. This *extended symptom profile* should include physical as well as behavioural indicators of hypersensitivity, discomfort and pain. Scratching and pain ought to be separated as two distinct entities. In particular, the distinction between scratching with and without skin contact should be encompassed in the extended symptom profiling, as should spontaneous and evoked vocalisation. A more specific clinical phenotype may be provided if it includes the localisation of scratching and pain and a distinction between the two symptoms. To secure inclusion of dogs with the well-defined phenotype for use in future research, it is necessary to comply with strict classification criteria. The author of this thesis advocates that three classification criteria should be met to be included as a case with a tentative diagnosis of CNeP as illustrated in the Venn diagram in Figure 6: an extended symptom profile based on owner reported indicators of discomfort and pain, a standardised clinical examination that confirms the presence of relevant symptoms and rules out other causes of scratching and pain in addition to the MRI-confirmed presence of syringomyelia.

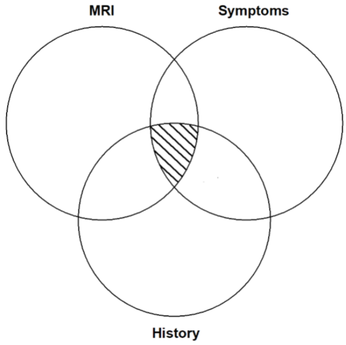


Figure 6 The well-defined phenotype definition of CKCS with central neuropathic pain and syringomyelia.
The Venn diagram illustrates the criteria that should be met to define a well-characterised CKCS with the tentative diagnosis of CNeP: *owner reported indicators of discomfort and pain* AND *a standardised clinical examination that confirms the presence of physical as well as behavioural indicators of hypersensitivity, discomfort and pain* where other causes of scratching and pain are ruled out AND *the MRI-confirmed presence of syringomyelia*.

Experimental animal models of central neuropathic pain

More than 20 rodent models of neuropathic pain are available (Burma et al. 2017). Most of these are peripheral nerve injury models, where the injury is inflicted by ligation, chronic constriction or transection of a peripheral nerve. Induction of diabetes, administration of chemotherapy, anti-HIV medications, alcohol and infection with varicella zoster virus in rodents are used to model metabolic, chemically induced and disease-specific peripheral neuropathic pain (Burma et al. 2017; Kumar et al. 2018). In models of CNeP, spinal cord injury is caused by compression, contusion or hemisection. A more specific localised spinal cord lesion can be inflicted by microinjection of excitotoxic quisqualic amino acid. Spinal ischemia is modelled by ligation or by an intravascular, laser beam-induced photochemical activation of the photosensitising dye erythrosine B. Demyelination pain in multiple sclerosis is modelled by experimental induction of viral or autoimmune encephalomyelitis (Kumar et al. 2018).

Despite attempts to refine such models, few mimic the clinically relevant human neuropathic phenotype (Klinck et al. 2017). Most of these models represent neuropathological changes during the *acute* onset of pain (Yezierski & Hansson 2018). The models enable in vivo studies of the structural and functional consequences of the inflicted nervous system lesion. The lesions inflicted on the nervous system may result in hypersensitivity. This hypersensitivity is assessed by applying standardised mechanical and thermal stimuli. The endpoint is an evoked limb withdrawal. Hence, the hypersensitivity is characterised by increased response or lowered thresholds to the stimuli. This reflex-behaviour is translated into the complex clinical manifestations of neuropathic pain in humans, who are experiencing allodynia and hyperalgesia (Rice et al. 2018). In addition, the relatively short duration of most preclinical investigations is far from being representative of the clinical situation. Here, for example in the case of syringomyelia, the development of neuropathic pain may be of slow progression due to degeneration rather than an acute trauma.

The translational gap

The analgesic efficacy of a potential new compound is likewise assessed by evaluating the animals' responses to stimuli. A pain-relieving effect of the compound is inferred by increased threshold or less responsiveness after pharmacological intervention (Honore et al. 2011; Rice et al. 2018). However, the measurement of this evoked limb withdrawal that reflects hypersensitivity, only represents the specific group of human neuropathic pain patients with sensory gain and allodynia or hyperalgesia, which affects the validity of the models (Berge 2011; Percie du Sert & Rice 2014). In human neuropathic pain patients, the pain phenotype is characterised by verbal descriptors of pain in addition to sensory characterisation of neuropathic components including allodynia and hyperalgesia. Changes in the somatosensory profile in terms of diminished or ceased allodynia and hyperalgesia after pharmacological intervention have been proven in human clinical trials, and have therefore been used as outcome measures (Bouhassira et al. 2014; Jensen & Finnerup 2014). However, the somatosensory disturbances change over time, and human patients with syringomyelia related CNeP far from always present identical sensory profiles (Ducreux et al. 2006; Hatem et al. 2010). Hence, a translational gap exists between the experimental animal models of pain and the experienced pain in the human target population. The consequence is a stagnation in drug development (Rice et al. 2018; Yezierski & Hansson 2018). Compounds that are found effective in pre-clinical studies often fail in clinical trials. The present likelihood is only 10.7% that a new analgesic compound will be approved by the American Food and Drug Administration (Hay et al. 2014; Finnerup et al. 2015; Yekkirala et al. 2017). Accordingly, the relevance and validity of the existing experimental models have been questioned (Berge 2011; Rice et al. 2018).

Desiderates of future animal models of neuropathic pain

Desiderates to relevant alternatives to the homogeneous rodent models have been put forward (Burma et al. 2017; Rice et al. 2018). Amongst other things, future models should:

- Represent the actual target population including the variability between individuals with regard to sex, age, genetic and environmental background, such as social interaction and co-morbidities (Rode et al. 2007; Burma et al. 2017; Yezierski & Hansson 2018).
- Mimic the clinical transition from acute to chronic pain and the onset and maintenance of neuropathic pain (Burma et al. 2017; Rice et al. 2018).
- Represent a specific disease with a specific aetiology pertaining to a human neuropathic pain condition (Rice et al. 2018) or
- Reflect the complex neuropathic pain phenotype of human neuropathic pain across aetiologies (Rice et al. 2018; Yezierski & Hansson 2018).
- Enable classification and stratification into representative subgroups of different pain phenotypes (Baron et al. 2017; Rice et al. 2018).
- Express measurable, non-evoked efficacy parameters that are clinically relevant and transferable, to increase the predictability of a given compound's usefulness in the clinical setting (Burma et al. 2017; Yezierski & Hansson 2018).

The translational potential of CKCS with symptomatic syringomyelia

The variability within the population of pet dogs more closely resembles the variation in the human target population with regard to age, sex and genetic background compared with rodent models that often comprise young males of the same strain only (Kol et al. 2015). In addition, by living in a family, the dogs are under influence by physical and social environmental factors similar to humans (Klinck et al. 2017). Finally, the life-span of the pet-dog is considerably longer than the life-span of rodents, which enable long-term follow-up (Klinck et al. 2017).

Spontaneous syringomyelia in dogs and humans share a number of common characteristics. The concomitant Chiari-malformation serves as an aetiological explanation for the development of syringomyelia in both species. Cerebellar herniation in dogs and the caudal descent of cerebellar tonsils in humans may result in abnormal CSF flow leading to syrinx formation. Also, symptomatic as well as asymptomatic syringomyelia related to Chiari-malformation is seen in both dogs and humans. The five-year follow up in Study I revealed that the clinical phenotype is stable over time. When symptomatic syringomyelia has evolved, the dog remains symptomatic. Hence, affected dogs are accessible in relevant numbers for future studies compared with the relatively few human cases. As shown in Study III, a number of histomorphological characteristics are common in both dogs and humans with symptomatic syringomyelia. The CNS lesions result in structural lesions of at least two constituents of the ascending sensory pathway: the superficial layers of dorsal horn grey matter and DREZ. The pathogenesis, however, remains incompletely understood in both species (Hechler & Moore 2018).

Should the CKCS with symptomatic syringomyelia function as a spontaneous model in clinical trials evaluating potential new active compounds, the clinical characterisation should preferably be extended by a quantitative sensory assessment. This would enable the desiderated classification and stratification into representative subgroups with specific sensory profiles as proposed by Rice *et al.* (2018). Despite the attempt to quantify the dogs' MST in Study II, it was not possible to demonstrate conclusively whether symptomatic dogs have altered MSTs. The feasibility and reliability of mechanical and thermal sensory quantification have already been evaluated in healthy, client owned dogs and reported in two studies (Sanchis-Mora et al. 2017; Ruel et al. 2018). However, the moderate repeatability and reliability of the current quantitative sensory test-protocols, together with the variability between dogs due to age, weight and sex, mean that quantitative sensory testing cannot replace a thorough history uptake and clinical examination (Hunt et al. 2019). Hence it remains unproven whether quantitative sensory assessment can be implemented in the clinical characterisation of symptomatic CKCS with syringomyelia.

Scratching, the primary outcome assessed in Paper IV, is not a symptom reported by human patients with syringomyelia-associated neuropathic pain. However, as previously discussed, there is an overlap between how the nervous system processes itch and pain. It remains unknown whether the scratching in CKCS with syringomyelia is a physical indicator of pain and discomfort. However, as shown in Study IV, PGN is proven effective to reduce the clinical signs of CNeP including scratching in the CKCS with syringomyelia. Though the therapeutic action of PGN in neuropathic pain is incompletely understood (Alles & Smith 2018), gabapentinoids are first-line recommendations for treating human neuropathic pain (Finnerup et al. 2015). Likewise, gabapentinoids are found effective in the treatment of certain chronic itch conditions including neuropathic itch in humans (Chuquilin et al. 2016; Pongcharoen & Fleischer 2016; Andersen et al. 2018). Since PGN is effective in human neuropathic patients as well as in experimentally induced models of neuropathic pain, the results of Study IV support the back- and forward translational potential of this spontaneous model (Rode et al. 2006).

Different hypotheses require different models, and no single animal model can perfectly reflect the human neuropathic pain condition one-to-one. Probably, the direct translation of symptoms and characteristics of human neuropathic pain into animal models is unattainable. Nevertheless, pet-dogs with symptomatic syringomyelia constitute a highly useful counterpart to the rodent models with regard to several key elements: the spontaneous development of disease, the gradual outbreak of symptoms, a slow progression over time and a diverse clinical phenotype. In conclusion, the author of this thesis proposes that the CKCS with symptomatic syringomyelia offers a superior model of neuropathic pain that fulfils several desiderates for future research compared with rodent models. In addition to study the pathogenesis of syringomyelia per se, investigations on the early outbreak of symptoms, the transition from symptom debut to the chronic state and the long-term outcome in affected individuals is possible. It would further strengthen this model's translational potential if the causal relation between syringomyelia and neuropathic pain were to be established.

Perspectives

Future investigations of spinal cord tissue from clinically well-characterised symptomatic and asymptomatic CKCS may elucidate, why some individuals with syringomyelia develop symptoms of CNeP while others do not. In-depth quantitative and histopathological assessment can provide novel information about the causal relationship between syringomyelia and neuropathic pain at the cellular and molecular level. It would be relevant to investigate whether excitotoxic neuronal necrosis or apoptosis is involved in grey matter loss. This can be done by quantification of immunolabelled sub-populations of GABAergic and glycinergic dorsal horn interneurons and caspase 3 or TUNNEL-positive neurons respectively (Polgar et al. 2005). In addition, the immunolabelling of interneurons would reveal the possibility that a phenotypic shift appears in the GABAergic and glycinergic dorsal horn interneurons as an explanation of symptoms in affected dogs (Knabl et al. 2008; Braz et al. 2012). To clarify whether the microglial activation and cytokine-induced long-term potentiation of excitatory dorsal horn projecting neurons is involved in CNeP generation as proposed by Gwak *et al.* (2017), quantitative microscopic investigations of spinal cord tissue labelled for microglia cells (IBA1) and proinflammatory cytokines should be undertaken. Laser micro dissection combined with the Nanostring technology can be applied to isolate neurons and glial cells from the spinal cords in order to investigate and quantify specific neuropathological and inflammation biomarkers on a single cell level.

The high heritability of symptomatic syringomyelia of 0.81 in the Danish population of CKCS evidenced in Study I combined with available pedigree information on the dogs, offer valuable components for the generation of future hypotheses. There is a potential for identifying one or more candidate genes associated with symptomatic syringomyelia in the GWAS briefly mentioned on page 30. This in turn offers a future back- and forward translational potential. The back-translation would imply genetical manipulation of rodents. Experimental genetical modification of rodents may enable spontaneous development of disease. Investigation of the aethiopathogenesis and of the resultant phenotype - including the structural, functional, cellular and molecular consequences of syrinx formation - may identify new potential targets for drug development. The forward translation would consist of a comparative exploration of the candidate gene's relevance in relation to cause and consequence in human syringomyelia. Finally, application of targeted "next generation sequencing" to spinal cord material combined with laser micro-dissection enables investigation of specific polymorphisms and detection of mosaics of the candidate gene(s) identified in the GWAS. In favour of the pet-dogs, the identification of one or more candidate genes potentially leads to the identification of individuals and breeding combinations that are at high risk of disease development. This information can then be used to reduce the occurrence of symptomatic syringomyelia in the CKCS population.

Development and subsequent validation of *the extended symptom profile* assessment tools will enable standardised clinical characterisation of each individual patient. Such a development will also increase awareness of the specific symptoms and improve quotidian clinical decision-making with regard to treatment planning and evaluation of treatment effect. Furthermore, the proposed extended symptom profiling enables classification of dogs based on their symptomatic phenotype. In turn, this classification would permit the suggested stratification into representative subgroups of different clinical phenotypes (Baron et al. 2017; Rice et al. 2018). Phenotype alterations after pharmacological intervention may serve as an alternative biomarker in future clinical trials. Future long-term assessment of non-evoked, discomfort- and pain-related behavioural outcome measures will elucidate, if other symptoms than scratching, phantom-scratching and vocalisation is pharmacologically sensitive in the CKCS with syringomyelia-related CNeP. Additionally, alignment in the clinical characterisation of cases enables the inclusion of a more homogeneous, and thereby easier comparable, study population within and between future studies. Moreover, this would enable alignment in outcome assessment in future clinical trials exploring the efficacy of TCAs or combination therapies shown to be effective in human neuropathic pain.

Conclusion

The present thesis has contributed to the current knowledge about neuropathic pain in Cavalier King Charles Spaniels with syringomyelia. Accordingly, the author has proposed the use of the Cavalier King Charles Spaniels with symptomatic syringomyelia as a spontaneous model of central neuropathic pain. It was shown that symptomatic syringomyelia occurs in the breed with a prevalence of 15%. The heritability is high, suggesting a strong genetic impact on the clinical phenotype. The hypothesized alteration in mechanical sensory threshold could not be confirmed as a descriptor of the clinical phenotype. An association was confirmed between the expression of symptoms and (1) syrinx diameter and (2) syrinx / spinal cord ratio, as was an association between a significant loss of dorsal horn grey matter and dorsal root entry zone degeneration. Spontaneous scratching, evoked scratching and vocalisation when scratching was found to be common quantifiable physical and behavioural indicators of hypersensitivity, discomfort and pain between the dogs. Finally, the model was proven effective to predict pregabalin’s efficacy for the reduction of clinical signs of neuropathic pain in the dogs. The histomorphological findings and the results of the clinical trial demonstrate the back- and forward translational potential of this spontaneous model of neuropathic pain to fill the gap between the induced rodent models and human patients.

Paper I

Prevalence and heritability of syringomyelia in Cavalier King Charles Spaniels and long-term outcome in symptomatic and asymptomatic littermates

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Prevalence and Heritability of Symptomatic Syringomyelia in Cavalier King Charles Spaniels and Long-term Outcome in Symptomatic and Asymptomatic Littermates

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Background: Syringomyelia (SM) is common in the Cavalier King Charles Spaniel (CKCS). Dogs with syringes express clinical signs or might be clinically silent.

Objectives: To investigate the prevalence and heritability of symptomatic SM, the association between clinical signs and magnetic resonance imaging (MRI) findings, and long-term outcome.

Animals: All CKCS registered in the Danish Kennel Club in 2001 (n = 240).

Methods: A cross-sectional questionnaire-based prevalence study validated by telephone interviews and clinically investigated clinical signs of SM. Dogs were 6 years at the time of investigation. A prospective observational litter study including clinical investigations, MRI and 5-year follow-up of symptomatic and asymptomatic siblings. Heritability was estimated based on the scale of liability in the study population and litter cohort.

Results: The cross-sectional study estimated a prevalence of symptomatic SM at 15.4% in the population. Thirteen symptomatic and 9 asymptomatic siblings participated in the litter study. Spinal cord syringes were confirmed in 21 of 22 littermates (95%). Syrinx diameter and mean syrinx : spinal cord ratio were significantly correlated with clinical signs ($P < .01$). Estimated heritability of symptomatic SM was 0.81. Symptomatic SM motivated euthanasia in 20%. Dogs with syringes, which expressed no clinical signs at the age of 6, remained asymptomatic in 14/15 cases (93%).

Conclusions and Clinical Importance: The prevalence of symptomatic SM is high and genetics have a high impact on clinical disease expression. Further investigations of factors influencing the outbreak threshold of clinical signs of SM are desirable.

Key words: Chiari-like malformation; Dog; Epidemiologic; Genetics; Magnetic resonance imaging.

Syringomyelia (SM) is a neurologic condition occurring in a hereditary form in the Cavalier King Charles Spaniel (CKCS), the Griffon Bruxellois, in other toy brachycephalic breeds as well as in humans.[1–4] The prevalence of SM in the CKCS has not been estimated because of a lack of epidemiologic studies investigating larger populations of dogs. SM is characterized by the development of fluid-filled cavities (syringes) within the spinal cord parenchyma and is associated with Chiari I malformation in humans and Chiari-like malformation (CM) in the CKCS.[2,5–11] The pathophysiology of CM involves a decreased caudal fossa volume with overcrowding of the craniocervical junction and caudal descend of the cerebellum into or through the foramen magnum.[5–15] The pathogenesis of syrinx formation and the association between CM and SM is not fully understood, but it is believed that a multifactorial etiology including a local obstruction of the subarachnoidal space and abnormal cerebrospinal fluid dynamics are involved.[5,10,12,16–19] Syringes are predominantly found in the cervical region of the spinal cord, but can form in multiple locations.[20]

In humans, the SM-associated damage to nociceptive and other sensory pathways of the spinal cord, causes pain to be a prominent feature in 50–90% of adult patients.[9,21,22] Other common clinical characteristics in humans with SM include dermatomal patterns of mixed loss of thermal sensitivity and paradoxical association of hypersensitivity as well as trophic changes with hyperhidrosis, glossy skin, coldness, and

Abbreviations:

CKCS	Cavalier King Charles Spaniel
CM	chiari-like malformation
DICOM	digital imaging and communications in medicine
DKC	Danish Kennel Club
DVCAS	Department of Veterinary Clinical and Animal Sciences
FOV	field of view
MRI	magnetic resonance imaging
NEX	number of excitations
NSAIDs	nonsteroidal anti-inflammatory drugs
OME	otitis media with effusion
SE	spin echo
SM	syringomyelia
T1 and T2W	T1 and T2 weighted
TE	time to echo
TR	time to repeat
TSE	turbo spin echo

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The study was conducted at the Department of Veterinary Clinical and Animal Science, University of Copenhagen, Denmark.

Poster presentation of preliminary results at the ECVN/ESVN 26th Symposium, September 26–28th 2013, Paris.

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paleness in humans.[9,23] Dogs with SM display characteristic behaviors, such as phantom scratching, unwillingness to be touched or groomed in the head and neck region, and in severe cases, paroxysmal pain manifestations with vocalization, intense scratching, rubbing, and circling on the floor. In dogs with SM, these behaviors have been associated with pain.[16,24–26] CKCS with a magnetic resonance imaging (MRI)-confirmed syrinx might be asymptomatic.[21] In symptomatic CKCS, a wide syrinx diameter and asymmetric distribution of the syrinx affecting the dorsal horn are strong predictors of pain.[25,27] Middle ear effusion (hyperintense material within the tympanic bulla probably analogous to otitis media with effusion [OME] in humans) is often present in the CKCS and may be detected on T2-weighted MRI scans.[28–30] The incidence of OME in CKCS has been estimated to 32–54%.[28–31] It has been debated if some of the behaviors associated with pain in CKCS could be related to OME, but this however remains controversial.[28,29,32,33] The heritability of SM has been estimated to be 0.37 based on MRI-confirmed syrinx findings in a cohort of 384 CKCS, whereas the heritability of symptomatic SM has not previously been investigated.[1]

The aim of this study was to estimate the prevalence and heritability of symptomatic SM in Danish CKCS. Furthermore, to investigate the association between clinical signs and MRI findings and report long-term outcome in a CKCS litter cohort including symptomatic and asymptomatic siblings.

Materials and Methods

The study was initiated in 2007, finalized in 2012, and conducted at the Department of Veterinary Clinical and Animal Sciences (DVCAS), University of Copenhagen, Denmark. The study population consisted of all CKCS born and registered in the Danish Kennel Club (DKC) in 2001. The DKC register lists dogs chronologically by date of registration, and to obtain a DKC studbook, dogs must be registered no later than 3 weeks after birth. The study was conducted in 3 phases.

Definitions

In this paper, the terms "symptomatic" and "asymptomatic" have been chosen for simplicity sake although the authors acknowledge that "symptomatic" is a subjective term inappropriate for animals. "Symptomatic" is here defined as being indicative of a specific disease (in this paper, the expression of clinical signs of SM).

Phase I—Prevalence Study

A cross-sectional design was used to estimate the prevalence of symptomatic SM. The study investigated clinical signs of SM in the study population (n = 240). Dogs born in 2001 (>6 years old) were chosen to secure that clinical signs of SM, if any, would have had their debut at the time of investigation. The first contact to the owners was established with a mailed letter distributed by the DKC. A short introduction of the investigation was accompanied by an invitation to answer an enclosed screening questionnaire with dichotomous alternative questions along with a semiopen component in which comments could be stated. The questions addressed the following clinical signs indicating SM: unilateral/bilateral episodic scratching of the head/neck region, phantom scratching of the head/neck region (the paw does not touch the skin), reluctance to tolerate touching and/or grooming of the head/neck region and/or resists wearing a collar. Signs are worsened if the dog becomes exited, agitated or both, and signs, which may be interpreted as pain often associated with the neck region.[10,34] The owners were asked to return their answers to the DKC in an enclosed stamped envelope along with a written consent confirming their participation and providing contact information. Owners of dogs expressing at least one of the signs listed, or who reported other clinical signs that could raise a suspicion of SM, were subsequently contacted by phone by the investigators and enrolled in an interview. An extended standardized questionnaire was used to provide further information and validate the answers given in the screening questionnaire. The telephone interview was performed by 2 investigators (CSEJ and RMLH), supervised by 2 veterinary neurologists (HG and MB), and the answers were subsequently scrutinized in collaboration with a senior veterinary neurologist (MB). Dogs qualifying as suspected symptomatic SM cases after validating telephone interview were invited to participate in a clinical investigation at the DVCAS Companion Animal University Hospital, including clinical and neurologic examination and standard hematology, biochemical, and thyroid profiles. Based on the definition of "symptomatic" as being indicative of a specific disease (in this case SM),[35] a final evaluation procedure served to define (clinical) symptomatic SM cases. The evaluation procedure included the clinical signs reported by the owners subsequently confirmed by the investigators and supported by the results of the clinical investigation (focused on excluding possible differential diagnosis).

Phase II—Litter Cohort Study

Eight litters were selected for further investigation of the association between clinical signs and MRI findings, based on pedigree information and the criteria that at least 1 sibling had been identified as a symptomatic SM case in the prevalence study. The owners were contacted by phone and interviewed using an extended standardized questionnaire addressing signs of SM to report the clinical status of the dogs at the time of inclusion. All dogs were invited to participate in the clinical investigation including an oral investigator-owner interview, clinical and neurologic examination, standard hematology, biochemical and thyroid profiles, and MRI of the brain and cervical spinal cord (C1–T1). Dogs presenting with a heart murmur were further evaluated with ECG and echocardiography.

Magnetic resonance imaging scans were performed using a 0.2 Tesla Esaote Vet-scan. The standardized protocol included T1-weighted sagittal images of the neurocranium and cervical spinal cord (C1–C3) (T1-spin echo [SE] with a field of view [FOV] of 14 cm, time to echo [TE] of 18 milliseconds, time to repeat [TR] of 480–560 milliseconds, slice thickness 4 mm, a slice gap of 0.4 mm, and a number of excitations [NEX] of 3), T1-weighted transverse SE sequences (FOV: 14 cm, TE: 18 milliseconds, TR: 930 milliseconds, slice thickness 5 mm, slice gap: 0.5 mm and NEX: 3) of the neurocranium, T2-weighted sagittal sequences (high resolution turbo spin echo [TSE], FOV 14 cm, TE: 80 milliseconds, TR: 2,800 milliseconds, slice thickness 4 mm, a slice gap of 0.4 mm and NEX: 2) and T2-weighted transverse TSE sequences (FOV 14 cm, TE: 80 milliseconds, TR: 2,800–2,820 milliseconds, slice thickness 5 mm, a slice gap of 0.5 mm and NEX: 2) of the neurocranium and cervical spinal cord (C1–C3). T1 and T2 matrix: 256 × 256. The dogs were placed in sternal recumbency, and the total examination time per patient was 75 minutes. Scans were transferred to the web-based DICOM image viewing system RemoteEye,[a] blinded and subsequently evaluated individually by 2 investigators (CSK & HB) and additionally by an experienced veterinary radiologist (UW).

T2 weighted images were used for syrinx measurements. A syrinx was defined as a fluid-filled cavity in the parenchyma with a diameter ≥2 mm within the spinal cord. A presyrinx was defined as edema in the spinal cord with a lesion diameter <2 mm with or without central canal dilatation. A SM-positive case was defined as a CKCS with a MRI-confirmed syrinx. Descriptive data included syrinx diameter, cranial limit of the syrinx, distribution of the syrinx (symmetric or asymmetric), and spinal cord diameter. The syrinx : spinal cord ratio was calculated. If more than 1 syrinx was detected, only the largest was used in the calculations. The hypothesis of no association between the expression of clinical signs and the presence of a syrinx was tested using Fisher's exact test. The hypotheses of no association between the expression of clinical signs and (1) syrinx diameter and (2) syrinx : spinal cord ratio were tested using Student's t-tests. Data were analyzed by the statistical software in Excel,[b] $P < .05$ was considered significant. It was recorded if CM, findings consistent with otitis media with effusion (OME), or both were present. CM was diagnosed when cerebellar indentation caused by the supraoccipital bone with or without cerebellar herniation into or through the foramen magnum was identified. In addition, it was recorded if the investigators initiated treatment after the clinical and MRI investigations. In 2007, the treatment used would typically be NSAIDs and/or furosemide (Furix[c]) alone or in combination and in grave cases, furosemide and prednisolone (Prednisolon DAK[c]).

Estimation of Heritability. The scale of liability (standard deviation from threshold) in the 2 populations described in phase I and II were used for the estimation of heritability according to methods described for threshold characters.[36] The applet available at http://www.ihh.kvl.dk/htm/kc/popgen/genetik/applets/heritt.htm was used for the calculations.

Phase III—5-year Follow-up (2012)

A follow-up telephone interview was conducted in autumn 2012 with the purpose of investigating long-term outcome for the now 11-year-old siblings participating in the 2007 litter cohort. The telephone interview was performed by 1 investigator (CLS) and supervised by a senior veterinary neurologist (MB) to secure a standardized interview. A standardized questionnaire was used to collect data regarding the status of the dogs including information addressing alive/deceased, possible clinical signs, possible progression or remission of clinical signs, development of new clinical signs since 2007, treatment, and for deceased dogs, time and cause of death.

Results

Phase I—Prevalence Study

Of 240 owners contacted by mail, 134 responded, giving a response rate of 56% (Fig 1A). Eleven responders were excluded (6 because the dogs were euthanized before the age of 3, and 5 dogs where questionnaires were returned incomplete), leaving 123 dogs (61 females and 62 males) to be included in the investigation. Nineteen owners reported 1 or more clinical signs of SM in their dogs (positive responders), whereas 104 owners reported that their dog expressed no clinical signs of SM (negative responders). After the subsequent telephone interview and validation procedure, the 19 dogs (positive responders) were categorized as suspected symptomatic SM cases. Fourteen owners agreed to let their dogs participate in the clinical investigation, which revealed no other diagnosis than SM, which could explain the signs expressed, by the dogs. Five dogs did not participate in the clinical investigation, but were evaluated to be symptomatic SM cases based on the expression of multiple signs known to be associated with SM,[16,19,24,37] and where the owners reported that there had been no clinical findings related to possible differential diagnosis such as eg, skin disease, ear infection, etc. Thus, based on the clinical signs reported and confirmed by the investigators, and the results of the clinical investigation, 19 dogs (11 females and 8 males) were finally included as symptomatic SM cases. The estimated prevalence of symptomatic SM in 6-year-old Danish CKCS born in 2001 was 15.4% (19/123), $CI_{0.95}$ [9; 22].

Phase II—Litter Cohort Study

Eight litters were selected for further investigation based on the criteria that at least 1 sibling had been identified as a symptomatic SM case in the prevalence study. The 8 litters comprised a total of 35 dogs (Fig 1B). Seventeen dogs expressed clinical signs of SM, whereas 17 dogs expressed no signs of SM. One dog was excluded as it had been euthanized at the age of 15 weeks because of severe seizures.

Twenty-two of 34 dogs (63%), including 11 dogs identified as symptomatic SM cases in the prevalence study, were allowed by the owners to participate in full clinical work-up including MRI. Ten owners declined to let their dogs participate in the MRI investigation, primarily because of concerns associated with anesthesia. One 5-year-old dog had been euthanized the year before because of expression of severe signs consistent with SM and could therefore not be investigated, and the investigators excluded 1 dog because of severe heart disease.

Thirteen of the 22 dogs (59%) were classified as symptomatic SM cases, whereas 9 dogs displayed no clinical signs of SM. MRI scans, however, revealed syringes in the cervical spinal cord in 21 of 22 dogs (95%); the remaining dog (expressing no clinical signs of SM) had a presyrinx (Table 1). Thus, the MRI investigation revealed that 8 dogs were clinically silent (asymptomatic SM-positive cases), although having a syrinx.

There was no statistical association between expression of clinical signs and the presence of syringes ($P = .41$). A positive predictive value of the MRI scan was calculated to 0.62 (the probability that a dog would express clinical signs if it had a syrinx). Syrinx diameter and syrinx : spinal cord ratio was evaluated on T2W transverse sequences in 19 dogs. Two dogs were excluded because the MRI studies were incomplete. The syrinx diameter varied from 0.3 to 0.8 cm. The total mean of the maximum syrinx diameter was 0.55 cm. The mean maximum syrinx diameter was 0.63 cm in symptomatic dogs and 0.4 cm in asymptomatic dogs. The data demonstrated that

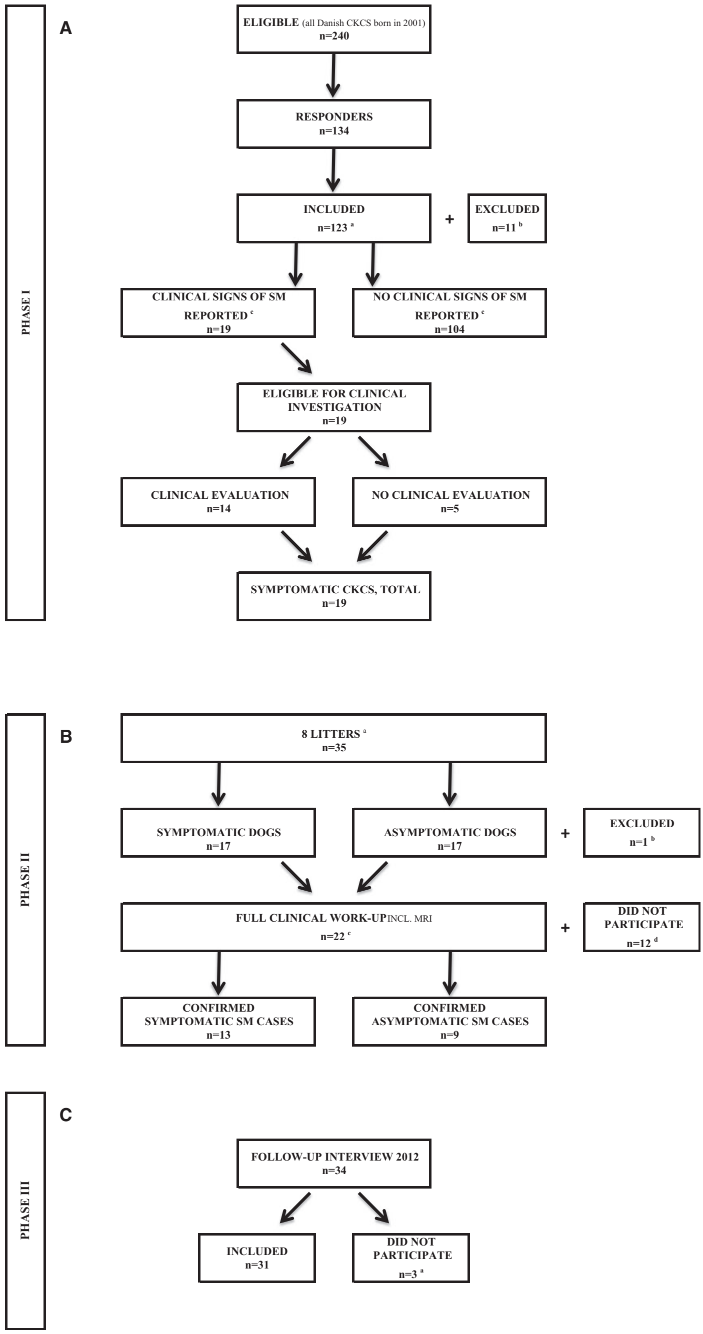

Fig 1. **(A)** Flow diagram, prevalence study—study phase I. [a]Six responders excluded because of euthanazia of the dogs before the age of 6, 5 because of incomplete questionnaires. [b]Sixty-one females and 62 males. [c]Clinical signs reported by the owners, validated on subsequent telephone interview. **(B)** Flow diagram, litter cohort study—study phase II. [a]Identified in study phase I (of all eligible Danish CKCS born in 2001). [b]Excluded because of euthanazia in 2001. [c]Including 11 dogs identified and validated in study phase I. [d]Ten dogs excluded because of reluctant owners, 1 because of euthanazia, 1 because of severe heart disease. **(C)** Flow diagram of the 2012 long-term follow-up study—study phase III. [a]Two owners declined to participate, 1 answer was excluded.

Table 1. MRI findings in 22 dogs participating in the litter cohort study.

	In All CKCS (n = 22)	In Asymptomatic SM Cases (n = 9)	In Symptomatic SM Cases (n = 13)	*P*-Value
Syrinx present/total (%)	21/22 (95)	8/9 (89)	13/13 (100)	
Syrinx diameter T2W (cm), mean (range)	0.55 (0.3–0.8)	0.4 (0.3–0.8)	0.63 (0.3–0.8)	.01
Spinal cord diameter (cm), mean (range)	0.94 (0.7–1.1)	0.9 (0.8–1.0)	0.96 (0.7–1.1)	.08
Syrinx : spinal cord ratio, mean (range)	0.58 (0.3–0.86)	0.43 (0.3–0.8)	0.66 (0.33–0.86)	.01
Asymmetric syrinx/total (%)	6/21 (29)	1/8 (13)	5/13 (38)	.19
Cerebellar herniation/total (%)	21/21 (100)	8/8 (100)	13/13 (100)	

MRI, magnetic resonance imaging; CKCS, Cavalier King Charles Spaniel; SM, syringomyelia; T2W, T2 weighted.

symptomatic dogs had a significantly wider syrinx diameter than asymptomatic dogs ($P < .01$). The total mean syrinx : spinal cord ratio was 0.58. Symptomatic dogs had a significantly greater mean syrinx : spinal cord ratio (0.66), when compared to asymptomatic dogs, which had a mean syrinx : spinal ratio of 0.43 ($P < .01$). The cranial limit of the syrinx was C2 in 17 of the 21 dogs (81%), C3 in 3 dogs (14%), and C1 in 1 dog (5%). Syrinx asymmetry (ie, unilateral dorsolateral deviation of the cavity) was observed in 6 (5 symptomatic and 1 asymptomatic) dogs. Fisher's exact test of the association between symmetry versus asymmetry and expression of clinical signs (yes/no) was nonsignificant ($P = .19$). CM was identified in 22 of 22 dogs (100%). OME was present in 11 dogs (5 symptomatic and 6 asymptomatic SM cases).

Estimation of Heritability. Heritability of symptomatic SM in dogs >6 years of age was calculated using information on difference in mean liability within the population and within related individuals to an affected animal, respectively.[36] Using the data presented (Table 2), the heritability was calculated at 0.81. In support of the high heritability, the difference between prevalence of symptomatic SM in the population and in the ones that were related to an affected full sibling was statistically significant (chi-square = 9.3; $df = 1$; $P < .05$).

Phase III—5-year Follow-up (2012)

Thirty-one of 34 dogs from the 2007 litter cohort (16 symptomatic and 15 asymptomatic dogs) participated in follow-up in 2012, where the dogs were 11 years old (Fig 1C). Two owners declined and 1 dog was excluded because of an incomplete interview. Eleven of the 31 dogs (35%) were alive, while 20 (65%) had been euthanized. Euthanasia was directly related to signs of SM in 4 dogs (20%). Thirteen of 16 symptomatic dogs remained symptomatic during the study period (Table 3A). With respect to expression of signs, 4 dogs experienced progression, 5 dogs remained status quo, 4 dogs experienced regression (3 with treatment) and 3 dogs became asymptomatic (1 with treatment) during the study period. Fourteen of 15 asymptomatic dogs remained asymptomatic during the study period, whereas 1 dog (with a MRI-confirmed syrinx in 2007) developed clinical signs of SM from 2007 to 2012.

When evaluating the 22 dogs participating in the clinical and MRI investigation in 2007 separately, the following results were obtained (Table 3B): Twenty of 22 dog owners participated in the 2012 follow-up, including 11 symptomatic SM cases and 9 asymptomatic SM cases (8 dogs with a MRI-confirmed syrinx and 1 dog with a presyrinx). Ten of 11 symptomatic SM cases remained symptomatic, whereas 1 previously symptomatic SM case became asymptomatic during the study period (without treatment). With respect to the expression of clinical signs in the 10 dogs, which remained symptomatic, 4 dogs deteriorated, 3 dogs remained status quo, and 3 dogs improved (with treatment) during the study period. Eight of 9 asymptomatic SM cases remained asymptomatic, whereas 1 dog developed clinical signs of SM during the study period.

Discussion

This study estimated a prevalence of 15.4% of symptomatic SM in Danish CKCS born in 2001 (>6 years old) and demonstrated that genetic factors strongly influence the clinical expression of SM.[36] The study furthermore reported that only a minority of asymptomatic dogs with a MRI-confirmed syrinx at 6 years develop clinical signs later in life.

Table 2. Prevalence of symptomatic syringomyelia used for heritability estimation.

	No. Non-affected Individuals	No. Affected Individuals	Frequency
In the population	104	19	0.1544
In full sib families	17	9[a]	0.3461
In remaining population[b]	87	10	0.1031

[a]The 9 individuals represent the 17 affected dogs minus the 8 index cases/propositus used for the selection of the families.
[b]Used for calculation of chi-square.

Table 3. Long-term outcome in (A) 34 and (B) 22 symptomatic and asymptomatic siblings identified in the 2007-litter cohort study.

		2012			
	2007	Status Quo	Deterioration	Improvement	Unknown
A					
Symptomatic SM cases	17	5	4	7[a]	1
Asymptomatic SM cases	17	14	1[b]		2
B					
Symptomatic SM cases	12	3	4	4[c]	1
Asymptomatic SM cases	10	8	1[b]		1

[a]Three dogs changed status from symptomatic to asymptomatic.
[b]One dog changed status from asymptomatic to symptomatic.
[c]One dog changed status from symptomatic to asymptomatic.

The dogs under investigation were older than 6 years of age, which served to secure that clinical signs of SM would have evolved at the time of inclusion. For a cross-sectional study based on mailed questionnaires, a response rate of 56% is considered to be good.[38] It is possible that the prevalence estimate is biased by the fact that owners with experience of SM might be more inclined to reply, whereas others may neglect signs of SM. Had all 240 owners returned the mailed questionnaire and had no new symptomatic cases been identified; the minimum prevalence had been 7.9% (19/240). The prevalence estimate was based on owner-reported clinical signs of SM, a telephone validation procedure, and a clinical evaluation, which served to further confirm the presence of clinical signs and rule out potential differential diagnosis. Five of the 19 dogs, which contributed to the prevalence estimate, did not participate in the clinical evaluation, and although nothing in the dogs' history indicated other diagnosis than SM, this is a weakness to the study, as we cannot exclude that some dogs were included as false-positive cases. However, 11 of the 19 dogs (60%) which participated both in the prevalence study and litter cohort study all had a MRI-confirmed syrinx and this supports that the dogs which expressed clinical signs of SM were true SM cases.

This study identified a substantial number of clinically silent dogs although having a syrinx and having symptomatic littermates. Symptomatic SM cases had a significantly greater syrinx diameter and a larger syrinx : spinal cord ratio compared to the group of asymptomatic SM cases which confirms previous reports.[25]

Our data allowed us to estimate heritability of the expression of symptomatic SM in CKCS 6 years of age, which has not previously been done. The heritability of SM diagnosed by a single MRI diagnosis without follow-up on clinical status has been estimated at 0.37,[1] whereas heritability of symptomatic SM was estimated at 0.81 in this study. It is apparent that the development of a syrinx does not always result in a clinical expression of signs of SM, and thus there may not be a discrepancy between these results. Although the heritability estimate for symptomatic SM might be an overestimation because of the fact that there is a potential bias in the prevalence study (dog owners with affected dogs might be more inclined to participate), the heritability is clearly very high. Per definition heritability is measured by estimating the relative contribution of genetic and nongenetic differences to the total phenotypic variation in a population.[36] The estimated heritability of symptomatic SM indicates that genetics explain most of the total phenotypic variance in the population.

As this study found an equal number of symptomatic and asymptomatic dogs with OME, and CM was confirmed in all MRI scanned dogs, it was not possible to examine if CM, OME alone, or both could have contributed to some of the clinical signs reported by the owners.

Finally, we acknowledge that the results of this study are affected by the drawbacks inevitably associated with epidemiologic and clinical studies, where it is seldom possible to motivate all owners to allow their dogs to participate in all planned procedures. It lies within the design of the study that all dogs should undergo the same standardized investigations as described in details in the materials and methods section. Being an epidemiologic study where the investigators did contact the owners, we must however respect that in some cases, owners do not want to participate in full work-up. In that case, the investigators must thoroughly determine for each dog if it can be defined as a true case based on the collected information. We also acknowledge that some of the choices made in 2007 regarding the study design may have influenced the results, eg, that measuring syrinx size on T2W images obtained from a low field system was suboptimal.[39] MRI identification of a syrinx is presently the gold standard for diagnosing SM. The results of this study provides information which necessitate further examination of factors contributing to disease expression and progression, and to the threshold of outbreak of clinical signs.

Conclusions

This study of Danish CKCS estimated a high prevalence of symptomatic SM and found a high impact of genetics on the clinical expression of the disease. Dogs with a MRI-confirmed syrinx and clinical signs are at risk of euthanasia, whereas dogs with a MRI-confirmed syrinx which are asymptomatic by the age of 6, seem to have a good chance of never developing such signs. It is of interest to conduct further studies investigating factors, which may influence the threshold of outbreak of clinical signs of SM.

Footnotes

[a] See: http://www.neologica.it/html/RemotEye.php
[b] Microsoft Excel 2008 for Mac Version 12.3.6
[c] Takeda Pharma A/S, Langebjerg 1, DK-4000 Roskilde, Denmark

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Conflict of Interest Declaration: The authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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Paper II

Mechanical sensory threshold in Cavalier King Charles spaniels with syringomyelia-associated scratching and control dogs

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Mechanical sensory threshold in Cavalier King Charles spaniels with syringomyelia-associated scratching and control dogs

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ABSTRACT

It is assumed that Cavalier King Charles spaniels with Chiari-like malformation and syringomyelia experience central neuropathic pain. An association between spinal cord parenchymal lesions and specific clinical signs (e.g. spontaneous and evoked scratching, withdrawal, and paroxysmal pain manifestations with vocalisation) has been suggested. This led to the hypothesis that mechanical sensory threshold is altered in clinical cases. The aim of this study was to quantify the cervical mechanical sensory threshold using Semmes-Weinstein monofilaments in nine Cavalier King Charles spaniels with Chiari-like malformation and assumed syringomyelia-associated central neuropathic pain compared to eight control dogs. Clinical and neurological examination including magnetic resonance imaging was undertaken.

Mean mechanical sensory threshold was not significantly different between case and control dogs (*t*-test on log10 transformed data; $P = 0.25$). Substantial variation within and between dogs was seen, with individual thresholds ranging from 0.04 to 26 g in case dogs and from 0.02 to 10 g in control dogs. Based on these results, it is unlikely that Cavalier King Charles spaniels with Chiari-like malformation and syringomyelia have increased mechanical sensation characterised by lower mechanical sensory threshold when quantified with Semmes-Weinstein monofilaments. Whether clinical cases experience central neuropathic pain remains unknown. The assessment of sensory function in dogs with assumed central neuropathic pain should be multimodal and include not only mechanical but also tactile and thermal threshold quantification. The use of threshold quantification in a clinical setting is challenging due to an insufficient signal relative to the biological background noise within and between dogs.

Introduction

It is assumed that Cavalier King Charles spaniels (CKCS) with Chiari-like malformation (CM) and syringomyelia (SM) experience central neuropathic pain (Rusbridge, 1997; Rusbridge et al., 2007; Rusbridge and Jeffery, 2008; Plessas et al., 2012; Rutherford et al., 2012). Affected dogs express various clinical signs including spontaneous and evoked scratching, reaction to touch on the head and neck region and, in severe cases, paroxysmal pain manifestations with vocalisation (Sanchis-Mora et al., 2016; Sparks et al., 2018). This aberrant behaviour is suggested to result from sensory threshold alterations, resulting in dysaesthesia and allodynia, and it has been argued that there is an association between the structural lesions of the spinal cord parenchyma and sensory threshold alterations (Rusbridge et al., 2007; Rusbridge and Jeffery, 2008; Hatem et al., 2010; Hu et al., 2012; Plessas et al., 2012; Schmidt et al., 2013).

In humans, SM associated with CM has been described to cause central neuropathic pain as a consequence of the somatosensory nervous system lesions and abnormal spinothalamic function (d'Angers and Prosper, 1827; Spiller, 1923; Levy et al., 1983; Hatem et al., 2010; Jensen and Finnerup, 2014). The pathophysiological mechanisms underlying neuropathic pain are associated with spinal and supraspinal nociceptive hyperexcitability and central sensitisation (Latremoliere and Woolf, 2009; Cohen and Mao, 2014; Jensen and Finnerup, 2014). Somatosensory disturbances include dermatomal patterns of mixed loss of thermal sensitivity, paradoxical hypersensitivity and tactile

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allodynia (Gamache and Ducker, 1990; Hida et al., 1995; Ducreux et al., 2006; Hatem et al., 2010).

In humans, quantitative sensory testing is used in combination with clinical examination and questionnaires to assess the neuropathic pain phenotype by application of thermal, mechanical and tactile stimuli (Cruccu et al., 2010; Haanpaa et al., 2011; von Hehn et al., 2012). This validated psychophysiological measure of perception assesses the function of peripheral myelinated and unmyelinated sensory fibres and nociceptive pathways of the central nervous system. The subject's sensory threshold for heat and cold is assessed by using a thermal probe; the threshold for tactile stimuli is assessed using monofilaments; for pinprick sensation weighted needles are used and sensitivity to vibration is assessed by the application of a tuning fork or a vibrameter (Greenspan, 2001; Cruccu et al., 2010; Thaisetthawatkul, 2014).

In dogs, a number of studies have investigated the use of monofilaments and handheld electronic devices for mechanical sensory threshold (MST) quantification. The response has been evaluated in healthy dogs and in dogs with acute, nociceptive pain and to assess the analgesic effect of systemic ketamine, opioids and local analgesics (Duque et al., 2004; KuKanich et al., 2005a,b; Fitzpatrick et al., 2010; Case et al., 2011; Hardie et al., 2011; Kukanich and Papich, 2011; Pieper et al., 2011; Brydges et al., 2012; Moore et al., 2013; Sanchis-Mora et al., 2017). However, the use of MST quantification to evaluate somatosensory function and the relationship between sensory threshold and behavioural indicators of pain in dogs with anticipated central neuropathic pain has not previously been reported. The primary aim of this study was therefore to quantify the cervical MST with Semmes-Weinstein monofilaments in CKCS with assumed mechanical threshold alterations and central neuropathic pain associated with SM. We hypothesise that the structural spinal cord parenchymal lesions result in a significant difference in MST between CKCS with syringomyelia-associated scratching and MRI-confirmed SM-negative control dogs without clinical signs.

Materials and methods

A prospective case-control study was conducted at the University Hospital for Companion Animals' Referral Neurology Clinic, Department of Veterinary Clinical Sciences, University of Copenhagen. Client-owned, pedigreed CKCS were enrolled in the study between 19 December 2013 and 21 September 2015. The study was approved by the Danish Animals Experiments Inspectorate (Approval date 12 January 2015; Approval number 2015-15-0201-00456) and the local Ethics and Administration Committee (Approval date 28 January 2014; Approval number 2014-5).

Initially, the standardised questionnaire published by Rutherford et al. (2012) was used to screen all dogs before enrollment in the study and to establish the individual dog's neuropathic pain score. Owners were interviewed by the principal investigator about their dog's general health status, medical history, clinical signs, behaviour, and quality of life. Enrollment criteria for case dogs included uni- or bilateral spontaneous scratching directed at the neck or shoulder. Enrollment criteria for control dogs included age >5 years. The rationale behind the conservative age-limit was to ensure that control dogs were truly SM-negative. To ensure the inclusion of control dogs without altered nociception or chronic pain, potential control dogs were excluded if they had a previous or present history of neurological or musculoskeletal conditions, skin- or ear conditions causing pruritus, had undergone surgery or received anti-epileptic medications. Gestating or lactating bitches and potential control dogs treated with analgesics or anti-inflammatory agents <6 weeks prior to enrollment were excluded.

All dogs were assessed by the principal investigator and underwent a clinical and neurological examination including otoscopy and ear swab cytology, urinalysis, haematological, biochemical and thyroid profile assessment. Dogs were excluded if the clinical evaluation revealed dermatologic conditions causing pruritus, neurological or musculoskeletal conditions causing pain or conditions contra-indicating anaesthesia.

Mechanical sensory threshold quantification

The MST was quantified with Semmes-Weinstein monofilaments by the principal investigator after clinical and neurological examination and before MRI. To prevent discrepancies between owner-reported clinical signs, and clinical, neurological, and MRI findings and MST test results due to potential disease progression, the time between the interview with the owner and the clinical examination including MST quantification and MRI was <14 days.

To reduce the variation between dogs, the principal investigator examined all dogs under the same conditions in the same standard examination room where there was background noise related to a normal teaching and referral hospital environment. The dog was placed standing on an examination table. MST quantification was video recorded using a smartphone (iPhone 6s, Apple). One of three research assistants performed the video recordings sitting on a rolling stool in front of the table to follow the dog's movements without losing focus on the dog's face. Owners were placed behind the camera operator to maintain the dog's attention towards the camera. No sensory inputs beside the applied filament were allowed, and after each time the dog's position was corrected, there was a 10 s pause before resuming the threshold quantification.

Three testing points were defined at the lateral aspects of the neck innervated by the spinal cord segments C1–C6 (Fig. 1). The initial stimulus side (left or right side of the neck) was chosen at random by throwing a dice (simple randomisation). Twenty Semmes-Weinstein monofilaments (Touch Test 20 Piece Full Kit, North Coast Medical) ranging from 0.008 to 300 g could be applied in ascending order, until one of five predefined behavioural responses was elicited (attentional shift towards the stimulus, body twitch, headshake, evoked scratching and avoidance/withdrawal response; Table 1).

Stimulation was initiated at testing point I cranial to the spina scapula (Fig. 1). The filament tip was placed in contact with the skin and a light pressure was applied until the filament flexed. Each stimulus lasted 3 s followed by a 10 s interval. If no response was elicited by the applied monofilament at testing point I, the same monofilament was applied to testing point II and – if no response – to testing point III. If no response was elicited with the finest monofilament (i.e. 0.008 g) at any of the testing points I–III, the procedure was repeated with the next finest monofilament (i.e. 0.02 g). Increasing monofilament diameters were used until one of the five predefined behavioural responses was elicited. After a 10 s pause, the procedure was then repeated with the same monofilament to ensure that the response was reproducible (i.e. that two consecutive stimuli of the same intensity would elicit the same response). After threshold quantification on the initial stimulus side, the test procedure was repeated at the contralateral side of the neck.

The video recorded MST quantification was assigned a random number using random permutation before it was saved in a database. To ensure masked evaluation, the videos were evaluated without sound by the principal investigator 4

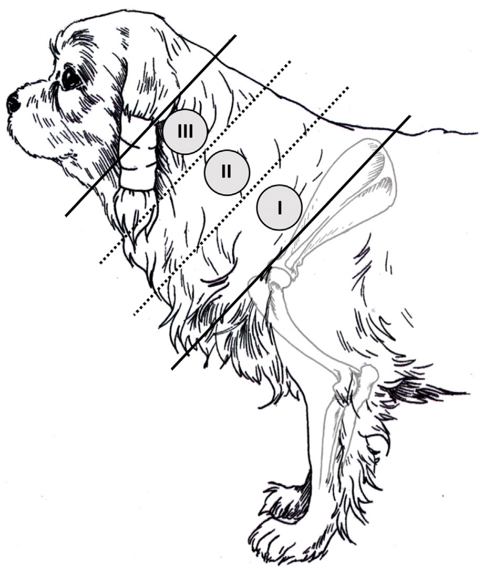

Fig. 1. Anatomical localisation of the testing points on the lateral aspects of the neck. To expose the testing points, the dog's pinnae were wound with VetFlex (Kruuse). The region of interest was visually outlined between two parallel lines – one defined by the spina scapula and the other parallel to the first line intercepting the wing of the atlas. The region of interest was then visually divided into three zones, with testing points I–III in the centre of these three zones (demarked I, II and III within the circles). Stimulations with Semmes-Weinstein monofilaments (range 0.008–300 g) were used to quantify the mechanical sensory threshold on both sides of the dog's neck and is reported as a mean of the two measures.

Table 1
An ethogram was designed for objective and standardised evaluation of possible behavioural responses elicited by stimulations with Semmes-Weinstein monofilaments in Cavalier King Charles spaniels with clinical signs of MRI-confirmed syringomyelia and in control dogs. If one of the five responses were elicited and reproduced, the filament size (g) was recorded.

Behaviour	Description of the dog's response when stimulated with the Semmes-Weinstein monofilament
Attentional shift	The head is turned towards the stimulus (>90° lateroflexion of the neck relative to straight forward)
Body twitch	The cranial part of the body is moved in a twitch
Headshake	Spontaneous headshake
Evoked scratch	The stimulus elicits a scratch episode
Avoidance/withdrawal response	A deliberate movement away from the stimulus

months after study termination. The primary outcome 'mechanical sensory threshold' was defined by the monofilament size in g that elicited a behavioural, reproducible response (Table 1). The sensory threshold was quantified on both sides of the neck and was reported as the mean of the two measures.

MRI protocol

Dogs were anaesthetised and placed in sternal recumbency. Case dogs underwent MRI (0.2 Tesla Vet-scan, Esaote) of the neurocranium and the spinal cord (C1–C6). The control dogs underwent MRI of the neurocranium from the interthalamic adhesion and as far caudal as possible but at least to the C4/C5 intervertebral disc space. Both protocols included T1-weighted (T1W) transverse, sagittal and dorsal sequences and T2-weighted (T2W) transverse and sagittal sequences. For specific details on the MRI protocol, we refer to the supplementary information (See Appendix: Supplementary MRI protocol). Scans were transferred to the web-based DICOM (RemoteEye, NeoLogica) and evaluated by an assessor masked to group allocation (UW). Syrinx evaluation and measurements were performed using transverse T1W MR images. SM was defined as a fluid-filled cavity in the cervical spinal cord parenchyma with a diameter $\geq$ 2 mm. CM was defined as cerebellar indentation with or without cerebellar herniation into or through the foramen magnum. The presence of T2 hyperintensity within the tympanic bulla led to the tentative diagnosis 'otitis media with effusion' (OME). The term OME will be used in this paper for simplicity although the authors acknowledge that the term is very specific and suggests a cellular diagnosis.

Statistical analysis

In a pilot study, dogs that had previously undergone clinical work-up for clinical signs associated with SM or pre-breeding MRI screening were invited to participate in MST quantification after clinical evaluation. In this pilot study, MST was quantified in 26 MRI-confirmed cases with SM-associated scratching and nine MRI-confirmed, SM-negative control dogs without clinical signs. Based on a normal distribution approximation and the assumption that the difference in mean MST between case and control dogs was 30% (standard deviation [SD] ± 20) with $\alpha = 0.05$ and $\beta = 0.8$, a minimum sample size of $n = 9$ in each group was calculated. To identify nine true negative control dogs, it was estimated that the required sample size was 20 CKCS without clinical signs associated with SM, since approximately 50% of all CKCS older than 5 years of age without clinical signs associated with SM are SM-positive (Parker et al., 2011).

To ensure that all data met the assumptions of parametric tests, MST data were log10 transformed before data analysis. In case of an outcome of 'no response to maximum stimulation with 300 g,' the numeric value of the MST was right censored and assigned '350' in the data set. Data was analysed using SAS Studio 3.71 (SAS Institute), with statistical significance set at $P < 0.05$. To test the hypothesis of no difference in mean MST between case and control dogs, an unpaired, two-sided t-test was applied. For dichotomous outcomes, Fisher's exact test was applied.

Results

Nine CKCS older than 1 year of age with clinical signs associated with MRI-confirmed SM were included as cases. Of 20 potential control dogs without clinical signs associated with SM that underwent clinical work-up, eight CKCS (40%) > 5 years of age were included as MRI-confirmed negative control dogs. Twelve dogs without clinical signs were excluded after MRI, because they were SM positive. The descriptive characteristics of case and control dogs are presented in Table 2. There was no significant difference in sex distribution ($P = 0.62$) and mean bodyweight ($P = 0.47$) between case and control dogs. The median age of case and control dogs was 3.34 years (interquartile range, IQR 0.92) and 7.23 years (IQR 1.83), respectively. The significant difference in age between the two groups ($P < 0.0001$) reflected the intentional age-specific selection.

The primary descriptive results of the owner interview are presented in Supplementary Table 1. Clinical evaluation revealed no dermatologic conditions causing pruritus, neurological or musculoskeletal conditions causing pain or conditions contra-indicating anaesthesia. Haematological, biochemical and thyroid profiles, urinalysis and ear swabs were unremarkable. None of the dogs had received analgesics within the previous 7 days before clinical evaluation and threshold quantification. The mean neuropathic pain score was 1.59 (range 1.0–2.29) in case dogs and 0.0 in all control dogs.

CM was present in all case and control dogs. The cases had a mean syrinx:spinal cord ratio of 0.53 ± 0.14 (range 0.29–0.71). The cranial limit of the syrinx was localised within the C3 segment in four of nine case dogs (44%). Six case dogs presented with asymmetric syrinxes. Spontaneous scratching was unilateral and directed to the same side as the asymmetric syrinx in four of the six case dogs (67%). In the three case dogs with symmetric syrinxes, the spontaneous scratching was unilateral (see Appendix: Supplementary Table 1). OME was found in three of nine case dogs (56%) and in five of eight control dogs (63%). The occurrence of OME (unilateral, bilateral or none) was not significantly different between the two groups ($P = 0.35$).

Mechanical sensory threshold quantification

The median MST was 0.9 g (IQR 175.1) in case dogs and 0.24 g (IQR 5.1) in control dogs (Table 3a). Mean MST was not significantly different between case and control dogs when comparing log10 transformed data ($P = 0.25$). In case dogs, a reproducible behavioural response (Table 1) was elicited by filaments sized between 0.04 and 26 g. In control dogs, filaments sized between 0.02 and 10 g elicited a reproducible behavioural response.

No significant differences were found between case and control dogs when the results of initial mean threshold quantification ($P = 0.09$) and contralateral mean threshold quantification ($P = 0.99$; Table 3a) were compared. Mean threshold did not differ between case dogs with symmetric and asymmetric syrinxes ($P = 0.54$; Table 3b). When comparing initial vs. contralateral mean thresholds, no differences were found within the control group ($P = 0.75$) or within the case group ($P = 0.06$; Table 3c and d). A comparison of mean thresholds for affected and non-affected sides in case dogs with unilateral scratching also revealed no difference ($P = 0.37$).

Table 2
Demographics of the 17 Cavalier King Charles spaniels included as case and control dogs, with continuous variables reported as median (range [interquartile range]), and categorical variables reported as n (%).

	Cases ($n = 9$)	Controls ($n = 8$)
Age (years)	3.34 (1.67–5.74 [0.92])	7.23 (5.62–9.13 [1.83])
Sex		
Female	6 (67%)	5 (63%)
Female spayed	1 (11%)	0 (0%)
Male	2 (22%)	3 (37%)
Male castrated	0 (0%)	0 (0%)
Bodyweight (kg)	9.4 (7.1–10.9 [1.8])	8.5 (5.8–10.2 [3.13])

Table 3
The mean of each dog's paired measurements on both sides of the neck represents the primary outcome mechanical sensory threshold (MST; g) and is reported here as median (range [interquartile range]) MST, in case and control dogs (a); within case dogs with symmetric and asymmetric syrinxes, respectively (b); and with the sub-group analysis comparing initial versus contralateral MST within control dogs (c) and case dogs (d).

(a)	Cases	Controls	*P*
Median MST	0.9 (0.06–175.7 [175.1])	0.24 (0.02–175.2 [5.1])	0.25
Initial MST	0.6 (0.04–350 [349.8])	0.16 (0.02–10 [0.3])	0.09
Contralateral MST	0.4 (0.04–15 [1.3])	0.07 (0.02–350 [5.3])	0.99
(b)	Cases, symmetric syrinx	Cases, asymmetric syrinx	*P*
Median MST	20.5 (0.1–175.2 [175.1])	0.6 (0.06–175.7 [175.1])	0.54
(c)	Controls, initial MST	Controls, contralateral MST	*P*
Median MST	0.16 (0.02–10 [0.3])	0.07 (0.02–350 [5.3])	0.75
(d)	Cases, initial MST	Cases, contralateral MST	*P*
Median MST	0.6 (0.04–350 [349.8])	0.4 (0.04–15 [1.3])	0.06
Symmetric syrinx	26 (0.04–350 [350])	0.4 (0.2–15 [18.8])	0.55
Asymmetric syrinx	0.5 (0.07–350 [349.8])	0.2 (0.04–1.4 [1.4])	0.18

Threshold responses were most frequently elicited at testing point I in case dogs and at testing point II in control dogs (in 50% and 63%, respectively, of all stimulations that led to a behavioural response). The MST quantification test procedure was well tolerated and completed in all dogs. The mean duration of the procedure (8 min 40 s in case dogs and 4 min 39 s in control dogs) was not significantly different between the groups ($P = 0.054$).

Discussion

This study investigated the hypothesis that spinal cord parenchymal lesions in CKCS with syringomyelia-associated clinical signs and MRI-confirmed SM would result in MST measurements that were significantly different from those in control dogs. Since tactile allodynia is one of the most commonly observed evoked pains in human patients with SM (Hatem et al., 2010), we expected to demonstrate lower thresholds as an indication of increased sensation in the case dogs. Despite a very strict and detailed study protocol and a very consistent testing procedure, we were unable to demonstrate the expected difference between case and control dogs. Subgroup analysis within the control dogs showed no differences in MST between the side of the neck used for initial stimulation and the contralateral side. This finding is consistent with previous findings in MST quantification studies in healthy dogs (Sanchis-Mora et al., 2017) and healthy humans (Rolke et al., 2006). Within the case dog group, the overall comparison of thresholds between the two sides of the neck revealed no differences. Additionally, we expected lower thresholds in case dogs with asymmetric syrinxes compared to those with symmetric syrinx distribution. We also expected that the thresholds in case dogs with unilateral scratching would be lower on the affected vs. the contralateral side, as previously reported in dogs with chronic pain (Brydges et al., 2012). However, subgroup analysis revealed no difference in MST related to either syrinx distribution or lateralisation of clinical signs.

Based on the results from our pilot study, we expected to detect a difference in MST between case and control dogs. Localisation and syrinx distribution, OME status and severity of clinical signs and neuropathic pain score are among the factors that varied between case dogs in the pilot study and those enrolled in the prospective study. The analgesic treatment had been discontinued by the owners in some of the pilot case dogs prior to MST quantification, and for most dogs this led to increased intensity of clinical signs and an assumed change in sensory threshold. These factors may have influenced the results of the pilot study which led to an inadequate sample size to detect a difference between groups in the present study.

In agreement with previous findings (Thofner et al., 2015), the prevalence of CM and OME was not different between dogs with and without clinical signs. Nevertheless, the authors speculate that CM and varying degrees of OME and coexisting deficiencies in eustachian tube pressure-equalisation could cause scratching directed at the ear, head and neck in some cases. Similarly, a recent study of owner-reported clinical signs in CKCS with CM–SM (Sparks et al., 2018) has suggested a possible lack of association between SM and the clinical pain phenotype, since CKCS with CM without SM can present with clinical signs previously reported to be associated with CM–SM (Rusbridge et al., 2007; Schmidt et al., 2013). The ideal control group would be CKCS without CM, SM and OME. The ideal case definition would accordingly have been a SM-positive CKCS with SM-associated clinical signs without CM and without OME. Unfortunately, the relative high frequency of both CM, SM and uni- or bilateral OME in CKCS expressing SM-associated clinical signs and clinically silent CKCS makes this almost impossible (Cerda-Gonzalez et al., 2009; Parker et al., 2011; Plessas et al., 2012; Sanchis-Mora et al., 2016).

In the search for a feasible and reliable diagnostic tool to identify dogs with altered MST and to evaluate treatment efficacy, our study was designed to mimic a clinically realistic situation. This may have increased the background noise relative to the signal. The methodological variation due to fluctuations in room temperature and individual (lack of) tolerance to distractions in and outside the room could have been reduced by using a quiet room at a constant temperature and by asking the owners to wait outside during threshold quantification. Variation in hair coat, thickness of the skin, dermal blood-flow and the distribution of cutaneous nociceptors are factors that have been reported to affect the sensory threshold quantification outcome in other species (Love et al., 2011). Hair coat variation has been accounted for in previous studies by hair clipping (Knazovicky et al., 2016; Sanchis-Mora et al., 2017). We decided to omit this due to the risk of skin trauma and local inflammation on the relatively large area of interest and the reluctance of owners, especially those who owned breeding and show dogs without clinical signs.

We chose three testing points on each side of the neck and decided that a reproducible response at any one site was enough to determine the MST of the given side of the neck. We also decided that the individual dog's MST should be reported as the mean of MSTs quantified on each side of the neck. The anatomical variation in lesion localisation among SM-positive CKCS (Loderstedt et al., 2011) was not accounted for in the protocol. Furthermore, the order in which the testing points were stimulated could have been randomised. It is debatable whether a mean of the MSTs quantified on each side of the neck is a representative measure of MST in individual dogs. In dogs that did not respond to maximum stimulation with 300 g, an arbitrary numeric value of 350 g was assigned (right censoring), which resulted in an arbitrary mean MST. Based on our experience, a better solution would have been to stimulate only one testing point on each side of the neck, or alternatively, to test the affected side in dogs with unilateral clinical signs. This approach would have increased the robustness of the dataset.

In human patients with neuropathic pain, a non-affected body area is chosen as a reference area to compare the quantitative sensory test results of the affected body area. This can be done because the patient is asked whether a specific body region is painful or not. The definition of a reference area in dogs is complicated due to the variability of the anatomical localisation of lesions in SM-positive dogs with clinical signs, and the veterinarian's lack of verbal communication with the dog. Moreover, a previous study (Williams et al., 2016) reported that C2 and C4 anatomical localisations produced different MST thresholds in SM-positive CKCS with and without clinical signs.

A major challenge was the evaluation of the dogs' responses to stimuli. The sensory threshold is a quantifiable measure, but the endpoint is determined subjectively, which can vary between observers and hence create bias. In a clinical setting, the risk of missing or misinterpreting a response is important. In the present study, the dogs' behavioural responses to stimuli were video-recorded and re-evaluated by the principal investigator masked to the group allocation of the dog in order to reduce inter-investigator bias.

MST quantification is one of several modalities used to assess and quantify sensory deficits. Studies of the complete somatosensory profile as described by Sanchis-Mora et al. (2017) should be undertaken to assess whether CKCS with clinical signs associated with CM–SM have altered sensory thresholds, which could indicate central, neuropathic pain. A thorough clinical characterisation of a larger cohort of case and control dogs would enable multivariate analysis and classification of dogs into distinct sensory subgroups. Since the completion of the present study, several other groups have published protocol validation and application studies (Harris et al., 2015; Freire et al., 2016; Gorney et al., 2016; Knazovicky et al., 2017; Sanchis-Mora et al., 2017). It is anticipated that these continuous research efforts will lead to optimisation of the testing protocol and hopefully enable methodological standardisation. Moreover, this should lead to further insights regarding how and where to quantify sensory thresholds, how to define a relevant reference area, how to interpret the responses to stimuli, and how to analyse and report the results.

Conclusions

The present study was unable to demonstrate a significant difference in MST between CKCS with SM and control dogs in spite of a very detailed and consistent testing procedure. The use of threshold quantification in a clinical setting is challenging due to an insufficient signal relative to the biological background noise within and between dogs. A thorough clinical characterisation and multimodal assessment of the somatosensory profile in a larger group of case and control dogs, and including not only mechanical but also tactile and thermal threshold quantification, would enable multivariate analysis to assess the effect of CM, OME, lesion-localisation and lateralisation of clinical signs on the somatosensory profile. It is still unknown whether CKCS with CM–SM experience central, neuropathic pain, but according to the findings in our cohort of dogs with MST quantified with Semmens-Weinstein monofilaments by our specific protocol, it is considered unlikely that CKCS with clinical signs associated with MRI-confirmed CM–SM have increased mechanical sensation.

Conflict of interest statement

This work was supported by The Danish Kennel Club and Dyrefondet. The funding sources played no role in the study design and in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication. None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tvjl.2019.01.011.

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Appendix A

Supplementary MRI protocol

Both protocols included T1- weighted (T1W) transverse images (T1-spin echo [SE] with a field of view [FOV] of 17 cm, time to echo [TE] of 18 milliseconds, time to repeat [TR] of 780-1000 milliseconds, slice thickness 4.5 - 5 mm, a slice gap of 0,5 mm, and a number of excitations [NEX] of 3-4), T1W sagittal SE sequences (FOV: 17 cm, TE: 18 milliseconds, TR: 490 - 560 milliseconds, slice thickness 4.5 mm, slice gap: 0,5 mm and NEX: 3-4), T1W dorsal SE sequences (FOV: 17 cm, TE: 18 milliseconds, TR: 490 milliseconds, slice thickness 4 - 4.5 mm, slice gap: 0,5 mm and NEX: 3-4), T2-weighted (T2W) transverse sequences (high resolution turbo spin echo [TSE], FOV 17 cm, TE: 80 milliseconds, TR: 3.090 - 3970 milliseconds, slice thickness 4.5 - 5 mm, a slice gap of 0,5 mm and NEX: 1) and T2W sagittal TSE sequences (FOV 17 cm, TE: 80 milliseconds, TR: 3.000 milliseconds, slice thickness 4.5 mm, a slice gap of 0,5 mm and NEX: 1). The T1 and T2 matrix was 256 x 256.

	Cases									Controls							
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8
Clinical manifestations of CM/SM (a)																	
Persistent compulsive scratching (b)	3	4	3	4	2	4	4	4	4	0	0	0	0	0	0	0	0
Facial rubbing	0	1	0	1	0	0	2	1	2	0	0	0	0	0	0	0	0
Hypersensitivity to touch	2	4	0	3	0	4	3	4	2	0	0	0	0	0	0	0	0
Unexplained yelping	2	0	2	1	2	1	2	2	3	0	0	0	0	0	0	0	0
Reluctance to lift the head	3	2	0	0	3	0	0	0	2	0	0	0	0	0	0	0	0
Reluctance to bend the neck to eat	2	2	0	0	3	0	0	0	3	0	0	0	0	0	0	0	0
Reluctance or pain when defaecating	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NeP score (c)	**2,00**	**1,86**	**1,00**	**1,29**	**1,43**	**1,29**	**1,57**	**1,57**	**2,29**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
NRS - scratching (d)	4 / 10	5 / 10	3 / 10	4 / 10	3 / 10	3 / 10	6 / 10	8 / 10	5 / 10	0	0	0	0	0	0	0	0
Scratching (uni- or bilateral / side) (e)	U / L	U / L	U / L	U / R	B	U / L	U / R	U / R	U / L	NO	NO	NO	NO	NO	NO	NO	NO
Owner perceived pain / discomfort																	
NRS - pain / discomfort (f)	N/A	1 / 10	4 / 10	N/A	9 / 10	3 / 10	6 / 10	3 / 10	6 / 10	0 / 10	0 / 10	0 / 10	0 / 10	0 / 10	0 / 10	0 / 10	0 / 10
Owner perceived quality of life (g)	3	3	2	4	3	2	3	3	4	1	1	1	1	1	3 (h)	1	1
Duration of threshold quantification (minutes:seconds) (i)	14:42	05:19	4:27	12:47	13:23	13:38	05:10	03:24	05:12	10:21	2:13	06:35	03:35	03:33	03:19	05:31	02:03
Mechanical sensory threshold [g]																	
Initial stimulus / side of the neck	350 / R	0.16 / R	0.04 / R	350 / R	350 / R	26 / R	0.4 / R	0.07 / L	0.6 / L	0.4 / L	0.07 / L	0.4 / L	0.16 / R	0.16 / R	0.16 / R	10 / R	0.02 / R
Contralateral side of the neck	0.4	0.07	0.16	0.4	1.4	15	1.4	0.04	0.07	350	0.04	10	0.07	0.6	0.04	0.07	0.02
Mean mechanical sensory threshold	**175.2**	**0.115**	**0.1**	**175.2**	**175.7**	**20.5**	**0.9**	**0.06**	**0.4**	**175.2**	**0.06**	**5.2**	**0.1**	**0.4**	**0.1**	**5.0**	**0.02**
Otitis media with effusion (uni- or bilateral / side) (j)	B	B	U/R	NO	NO	NO	NO	NO	NO	B	B	NO	NO	B	B	NO	U/R
Syrinx characteristics (symmetric, asymmetric / side) (k)	S	A / L	S	A / R	A / L	S	A / R	A / L	A / L								
Localisation (spinal cord segments affected)	C3 - C7	C2 - T1	C3 - C6	C3 - C5	C1 - C8	C3 - C5	C1 - C6	C1 - C4	C4								
Syrinx : spinal cord ratio	0.38	0.63	0.29	0.64	0.61	0.50	0.60	0.71	0.44								

Supplementary table 1. Descriptive clinical characteristics of the 17 CKCS included as cases (n=9) and controls (n=8).

(a) The clinical manifestations of CM/SM were described by the owners based on a systematic interview ad modum Rutherford et al. 2012. For all descriptors comprised in the neuropathic pain score (**NeP score**), the owners were asked to rate, to what degree they had observed the individual clinical manifestations. Possible answers on a five-point rating scale were: **0**=never, **1**=seldom, **2**=sometimes, **3**=usually, **4**=always.

(b) When interviewed about persistent compulsive scratching, it was emphasised that the scratching should not be caused by underlying skin disease. This was assessed and confirmed during clinical examination.

(c) **NeP score**: The neuropathic pain score is a result of the average across the individual clinical manifestation scores.

(d) **NRS, scratching**: Numeric rating scale from 0-10, where 0=no scratching and 10=worst possible scratching intensity, rated by the owners.

(e) **B**=bilateral, **L**=left side of the neck, **R**=right side of the neck, **U**=unilateral. The localisation of the dog's scratching was confirmed during clinical examination.

(f) **NRS, pain / discomfort**: Numeric rating scale from 0-10, where 0=no pain / discomfort and 10=worst possible pain / discomfort, rated by the owners.

(g) The owners were asked to rate their dog's quality of life on an eight-point rating scale, where 1=could not be better, 2=good, 3=fairly good, 4=neither good nor bad, 5=fairly poor, 6=poor, 7=could not be worse or 8=do not know.

(h) Due to regional semantic differences not unlike those between American and British English; as in the latter, the positive is often understated in the region of Denmark, where this owner resides.

(i) Time from mechanical sensory threshold quantification with Semmes-Weinstein monofilaments was initiated until a reproducible behavioural withdrawal response was elicited.

(j) The presence of T2 hyperintensity within the tympanic bulla led to the tentative diagnosis 'otitis media with effusion'. **B**=bilateral, **U/L**=unilateral, left side, **U/R**=unilateral, right side.

(k) Syrinx evaluation and measurements were done on transverse T1 weighted MR images. **S**=symmetric, **A / L**=asymmetric, left, **A / R**=asymmetric, right.

Paper III

Superficial dorsal horn volume loss in Cavalier King Charles Spaniels with neuropathic pain and syringomyelia – a quantitative and histomorphological characterisation of cervical spinal cord lesions

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Superficial dorsal horn volume loss in Cavalier King Charles Spaniels with neuropathic pain and syringomyelia – a quantitative and qualitative histological characterisation of cervical spinal cord lesions

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Conflicts of interest statement

The authors have no conflicts of interest to declare.

Disclosure

An abstract on the main results was accepted for poster presentation at the 7th International Congress on Neuropathic Pain in London, UK in May 2019.

Keywords

Cavalier King Charles Spaniels, Chiari malformation, dorsal root entry zone, SMI-32, spinothalamic pathway, stereology

Abstract

Syringomyelia give rise to central neuropathic pain in humans as well as dogs of the breed Cavalier King Charles Spaniel. A correlation between symptoms and magnetic resonance imaging findings (syrinx dimension, syrinx / spinal cord ratio and degree of syrinx asymmetry) has been established in both humans and dogs. However, description of histomorphological characteristics and their association with symptoms of central neuropathic pain in humans and dogs with syringomyelia are sparse. In this study, a quantitative and qualitative histological characterisation of cervical spinal cord lesions investigated 1) a possible relation between symptoms and volume loss of a specific anatomical entity involved in nociception and 2) if syringomyelia affected specific functional spinal cord units of nociception. Spinal cord segments C1-C8 from dogs with a well-characterised pain phenotype and MRI-confirmed syringomyelia (n=8) and asymptomatic controls without syringomyelia (n=4) were investigated. A significant volume loss (*P*=0.034) of the dorsal horn laminae I-III was found in the symptomatic compared to the asymptomatic half of the spinal cord in affected dogs after parametric, paired comparisons of stereological quantified volumes. A clear pattern of ipsilateral changes in the dorsal root entry zone characterised by deafferentation and reorganization of first-order axons into deeper laminae was found in cases with lateralised clinical signs. The loss of dorsal horn grey matter and dorsal root entry zone pathology are neurohistopathological characteristics shared with human syringomyelia patients. A henceforth confirmation of the causal relation between syringomyelia and CNeP in the CKCS potentially offers an analogy model to human syringomyelia-related central neuropathic pain with translational potential.

Introduction

Central neuropathic pain (CNeP) is defined as pain caused by a lesion or disease affecting the somatosensory system within the central nervous system (CNS) [1]. The most common diseases giving rise to CNeP in humans are: stroke, spinal cord injury and multiple sclerosis [2-4]. A rarer cause of CNeP is syringomyelia. Pain is the major complaint in up to 90% of human patients with syringomyelia [5-8]. Of these, up to 40% experience neuropathic pain characterised by dermatomal patterns of mixed loss of sensation and a paradoxical hypersensitivity [7, 9, 10]. A correlation between pain, abnormal sensations and magnetic resonance imaging (MRI) findings has been reported in adult human patients with syringomyelia secondary to Chiari-1 malformation [10, 11].

Clinical signs of CNeP are present in up to 15% of Cavalier King Charles Spaniel (CKCS) with cervical syringomyelia and Chiari-like malformation [12-15]. Common clinical signs are spontaneous or evoked unilateral scratching directed towards the neck region, avoidance of being touched or groomed and paroxysmal pain manifestations e.g. spontaneous vocalisation when the dog is physical active or when it is touched [16-18]. As in human syringomyelia, a correlation between clinical signs and MRI findings including syrinx / spinal cord ratio and the degree of syrinx asymmetry has been reported [12, 14].

In the normal functioning nervous system, peripheral sensory inputs are transmitted by first order neurons via the dorsal spinal nerves to the spinal cord's dorsal horns. The dorsal horn is organised in five laminae originally described by Rexed [19]. Nociceptive Aδ- and C-fibre inputs project to lamina I and the outer layer of lamina II in the superficial parts of the dorsal horn. Innocuous information is mediated by Aβ-fibres, and their axons predominantly synapse in laminae III and IV. The nociceptive information is transmitted via second-order neurons in the dorsal horn. The information is under powerful control of descending and segmental systems before subsequent transmission to third order neurons in the thalamus and other higher brain centres [20]. A CNS lesion may compromise the normal functions of this somatosensory afferent pathway. The lesion may cause negative symptoms with loss of Aδ- and C-fibre function. However, the lesion damaging these pathways may at the same time give rise to a series of positive phenomena with allodynia (=pain elicited by normally innocuous stimuli) and hyperalgesia (= increased pain produced by a noxious stimulus) [21].

The structural, functional and biochemical changes and their implication on nociceptive processing after CNS injury have been extensively studied in experimental animal models. However, descriptions of the histomorphological characteristics and their association with CNeP in humans as well as dogs with spontaneous occurring Chiari-malformation related syringomyelia are sparse [22-25]. In addition, the proposed biochemical and functional consequences of the CNS lesion and their relevance to clinical signs of CNeP are poorly understood. The present design-based stereological volume quantification was undertaken to elucidate, if one or more specific structural cervical spinal cord entities involved in nociception are affected by syrinx formation in CKCS with clinical signs of CNeP [26, 27]. A secondary aim was to assess, if a possible relationship between clinical signs consistent with CNeP and structural loss of a specific anatomical entity exists. Hence, a comparison of total volumes of the anatomical entities was made between the affected and non-affected halves of the spinal cord in cases with lateralised clinical signs of CNeP. In addition, a morphological assessment was undertaken to elucidate, if the syringomyelia-related lesions did affect specific functional spinal cord units of nociception.

Materials and methods

Twelve private-owned CKCS were included in this prospective case-control study between October 2014 and June 2018. The study was approved by the Department of Veterinary Clinical Sciences' local Ethics and Administration Committee (8th of August 2014, file no. 2014-5). The dogs had been enrolled in one or both of the Danish longitudinal observational studies in a cohort of CKCS with syringomyelia-associated CNeP and / or myxomatous mitral valve disease, and were donated by their owners after signed consent. Accordingly, the dogs were treated ethically as any other patient and under the terms of the Danish and European legislations on the protection of animals (Act no. 20 of 11th January 2018) and on animals used for scientific purposes (Directive 2010/63/EU).

Included cases (n=8) were MRI confirmed Chiari-like malformation- and cervical syringomyelia-positive dogs with a well-characterised pain phenotype as previously described [28]. Cases should express clinical signs of syringomyelia-associated CNeP characterised by scratching directed towards the neck region or with an evoked cervical pain focus confirmed on clinical examination that correlated to the MRI-findings. The dogs' syringomyelia-related clinical signs of CNeP had previously been well-controlled with different treatment regimens (Table 1). Due to disease progression and despite intensive efforts with add-on treatments, it was no longer possible to alleviate the symptoms and sustain an acceptable quality of life. Euthanasia was therefore decided for ethical reasons. Controls (n=4) were included if they were to be euthanised for any other reason than clinical signs of syringomyelia-related CNeP: two dogs due to severe myxomatous mitral valve disease, one due to bilateral patella luxation and one because of severe aberrant behaviour. Controls were excluded if they had a history of pain of more than 48 h duration, or if they had been treated with analgetics i.e. NSAIDs or opioids, antiepileptics or steroids within six weeks before euthanasia. Prior to euthanasia, the dogs' clinical and, for cases pain phenotype were characterised by the principal investigator (MST) based on a standardised interview *ad modum* Rutherford *et al*. [13]. Clinical and neurological examination including haemogram, biochemical and thyroid profile, urinalysis and otoscopy including ear swab cytology was undertaken in all dogs. Relevant descriptors are presented in Table 1.

Euthanasia, tissue sampling and preparation

At the time of euthanasia, the dogs were sedated by intramuscular (IM) injection of tiletamine (Virbac, Denmark) 1.25 mg/kg, zolazepam (Virbac, Denmark) 1.25 mg/kg and xylazine (ScanVet Animal Health, Denmark) 2 mg/kg except for the two controls with myxomatous mitral valve disease, who received dexmedetomidine (Orion Pharma Animal Health, Denmark) 0.025 mg/kg and butorphanol (Orion Pharma Animal Health, Denmark) 0.125 mg/kg IM. The dogs were euthanised by intravenous injection of pentobarbital (Virbac, Denmark) 400 mg/kg.

The spinal cord segments C1-C8 were sampled within 90 min after cardiac arrest as described by Agerholm [29]. Transection of the exposed spinal cord was guided by the anatomical landmarks of the region of interest: the most cranial and caudal emergences of the paired dorsal spinal nerves I and VIII respectively (Fig. 1). The length of the sampled spinal cord segments C1-C8 was measured before it was immersion-fixed in 4% phosphate-buffered formaldehyde (VWR Chemicals, Denmark) with a tissue to fixative ratio of 1:50 and kept at room temperature for 48 h. The weight of the cervical spinal cord was logged before dehydration in a Thermo Shandon Citadel 1000 tissue processor (Shandon Scientific Ltd., United Kingdom) and paraffin embedding (Histowax, Histolab, Denmark) in a custom-made metal mould. Additional information on cold ischaemic time, autopsy duration descriptors of the investigated spinal cord segments C1-C8 are presented in Table 2.

Immunohistochemical staining

To enable stereological volume estimation as described in detail below, the full-length paraffin-embedded spinal cord segments C1-C8 was cut into 5 mm tissue blocks. Systematic, uniform random sampling of the most rostral transverse section of each tissue block was undertaken with a section sampling fraction (SSF) of 1/2. The neurofilament triplet H protein-specific primary monoclonal mouse antibody, SMI-32 (BioLegend, Denmark) was used to identify and delineate the grey matter dorsal horns' laminae I-III [30]. Serial transverse 10 µm microtome sections (Leica RM 2255, Leica Biosystems, Germany) were mounted on TOMO slides (Histolab, Denmark) and left overnight at room temperature. After deparaffination (60 °C for 30 min) and rehydration (xylene for 20 min followed by graded alcohols in descending concentration from 99% to 96% to 70%, 10 min in each concentration before shortly rinsed in distilled H_2O). Endogenous peroxidase activity was blocked for 30 min with 30% H_2O_2 in tris-buffered saline (TBS). The slides were placed in target retrieval solution (Dako, Denmark), pH 6 and microwave-boiled at 80 °C for 10 min followed by cooling and rinse in 10% goat serum (Biowest, France) and 0.3% Triton X-100 (Merck, Germany) in TBS for 30 min. Incubation with the primary antibody, SMI-32 (BioLegend, Denmark) 1:100 – 1:1000 in 0.3% Triton X in TBS at 4 °C overnight. On the second day, slides were re-heated to room temperature followed by rinse in 10% goat serum (Biowest, France) and 0.3% Triton X (Sigma-Aldrich, Denmark) in TBS for 30 min before incubation with secondary polyclonal goat-anti-mouse HRP-conjugated antibody (Dako, Denmark) 1:400 for 2 h. An avidin-biotin complex (VWR International, Denmark) was used for detection before visualisation with 0.05% 3,3'-diaminobenzidine-tetrahydrochloride hydrate, DAB (Sigma, Missouri, USA) followed by dehydration for three times 10 min in graded alcohols of increasing concentrations, 70%, 96%, 99% and 20 min in xylene before final cover slip mounting. Human cerebellum and mouse brain slides were included in each batch as positive controls. Spinal cord from the same animal without incubation with the primary antibody was included as no-primary-control.

Histochemical stainings

Three additional section series were cut with sampling fraction 1/2. One series of 4 µm sections were mounted on Superfrost plus glass slides (Thermo Fisher Scientific, Denmark) and stained with haematoxylin and eosin. Two series of 30 µm sections were mounted on 1.3% gelatine coated Superfrost glass slides (Histolab, Denmark) and stained with luxol and thionine respectively.

Definition of the specific anatomical spinal cord entities involved in nociception

The serial transverse 10 µm microtome sections stained with SMI-32 were used to delineate the following anatomical spinal cord regions involved in nociception: The left and right dorsal horn laminae I-III as a representation of the 2nd order nociceptive neurons of the ascending pathway, areas of the white matter's dorsal, lateral and ventral funiculi as representatives of the dorsal and lateral spinothalamic columns and the ventral area of spinothalamic fibre decussation (Fig. 2).

Equipment

The individual whole slide tissue sections were viewed in the stereology software system newCAST™ (Visiopharm, Denmark) via a high-resolution digital Olympus DP71 camera mounted on an Olympus MVX10 Macro View (Olympus Corp., Center Valley, PA, USA). The 2D nucleator was applied for area estimation of the predefined areas on each cross section using eight test rays. A series of pilot-estimations were run in advance to decrease the estimate-variation, and a decision was made to use eight test rays for each area-estimation.

Volume estimation

The Cavalieri estimator [31-33] was applied for subsequent estimation of the total volumes of the regions of interest: the spinal cord segments C1-C8, the spinothalamic functional units, the remaining grey matter, the central canal and the total volume of tissue loss if any by means of the following formula:

$$V_{(\mathrm{TOTAL})} = \frac{1}{SSF} \cdot \Sigma A \cdot 2T$$

where SSF equals 1/2, A is the estimated cross section area on individual slides and T is the thickness of the tissue block (5 mm).

Morphological assessment of specific anatomical spinal cord entities affected by syringomyelia

Based on previous light microscopy histopathological descriptions of syringomyelia [22, 25, 34-36], a protocol was made for systematic assessment of all tissue slides. The widest diameter of all visible fluid-filled cavities within the spinal cord parenchyma were measured. The following measurements were used for classification: a diameter of ≥ 2000 µm: syrinx. Diameter ≥ 1500 to 2000 µm: pre-syrinx. A central canal dilation was defined as a cavity completely or partially lined with ependymal cells with a diameter ≥ 500 to 1500 µm. Moreover, the description of the cavities included: symmetry (yes / no) and if asymmetric, which dorsal quadrant was primarily affected (left / right). The shape of the cavity was categorised as either circular, oval, flattened, star-shaped, squared or slit-like (Fig. 3 [f]).

The relevant anatomical areas of the ascending nociceptive pathway were assessed to clarify the relationship between clinical signs of CNeP and structural damage. The neurohistopathological evaluation of the neuropil and parenchyma included an assessment of the presence or absence of 'pseudorosettes' described as ciliated ependymal cells around vessels by Hu *et al.* [22], oedema, gliosis, infiltration of leukocytes or lymphocytes or degeneration (for grey matter: in particular chromatolysis as an indicator of apoptosis; for white matter: spongiosis, swollen axons or lipid laden 'foamy' macrophages. The dorsal root entry zone (DREZ) appearance was evaluated. If an abnormal appearance of the DREZ was seen, it was classified as either disorganized (primary axons terminating in deeper laminae compared to the contralateral dorsal horn), degenerated (loss of axons in the DREZ) or not visualisable (absence of axons in the DREZ due to syrinx dissection through pia mater). Hereafter, images of all slides were digitalized by an Olympus VS110 Virtual Microscopy System (Olympus, Denmark), with a 20x NA 0.75 oil objective to enable an equivalent evaluation by a senior neurologist (TSJ) blinded to the clinical status of the cases.

Outcome measures and statistics

Descriptive statistics for categorial variables are reported by frequencies and proportions and for continuous data as medians and ranges. Data analysis included parametric comparisons of estimated mean total volumes between cases and controls (two-sided comparisons of the means of independent samples), and within cases with lateralised symptoms (paired, two-tailed t-tests) in SAS Studio 3.71 (SAS Institute Inc.; NC, USA). $P < 0.05$ was considered significant. Data from one dog with bilateral scratching were excluded from the within-cases comparisons.

Results

Stereological volume estimation

Overall comparisons of the total mean volumes of the spinal cord and sub-volumes revealed no significant differences between cases and controls (Table 3). The only significant difference between the two groups was the total volume of the central canal, 13 μm^3 (range 6 - 26) in controls vs 188 μm^3 (range 49 - 461) in cases (P=0.04).

A subsequent paired comparison of total volumes of the specific anatomical entities between the affected and non-affected halves of the spinal cord in cases with lateralised clinical signs of CNeP (n=7) was undertaken. A significant volume loss of the dorsal horn laminae I-III as a representation of the 2nd order nociceptive neurons of the ascending pathway was found on the affected side to which the clinical signs were ascribed: 34 μm^3 (range 20 - 48) compared to the non-affected side, 42 μm^3 (range 27 - 54, P=0.034). Additional comparisons of the total volumes of grey matter, grey matter laminae IV-X, white matter, dorsal, lateral and ventral columns were insignificant (Table 3).

Descriptive histomorphology

Syringomyelia was present in ≥ 5 cervical spinal cord segments in 4/8 cases, and the segments most frequently affected were segments C2-C4 (5/8 cases). The median syrinx diameter of 3135 µm (range 1227 - 4668) was significantly different from the median central canal diameter of 527 µm (range 419 - 803) in controls (P=0.0052). A central canal with intact ependyma without syrinx formation was seen in the rostral segments of 8/8 syringomyelia-affected spinal cords. The syringes were accordingly classified as non-communicating with the 4th ventricle [37]. When evaluating the cranial pole of the syrinx, the cavity formation was independent of the dilated central canal in 5/8 cases (Fig. 3 [a, f]). Moving caudally along the neuraxis, a fusion-pattern between the central canal and the cavity was seen in all the 5/8 cases (Fig. 3 [b, g]). The caudal pole of the syrinx was situated within the C1-C8 segments in 6/8 cases. The fusion-pattern between the central canal and the cavity formation ceased in all 6/8 cases, and a distinct central canal was identified in the most caudal segments of the spinal cord (Fig. 3 [d, h]).

In segments without a fusion between the central canal and the cavity formation the shape of the central canal remained either circular, oval, flattened, triangular, star-shaped or squared. In contrary, the concomitant incipient cavities were slit-like and dissected into the dorsal quadrant’s grey matter (Fig. 3 [a, c, f, h]). The slit-like pattern persisted after fusion between the central canal and the cavity. All cases with unilateral clinical signs of syringomyelia-related CNeP had asymmetric syringes. However, the previous reported association between lateralised symptoms and an asymmetric syrinx distribution to the ipsilateral, symptomatic side on MRI [12] could not be confirmed. The distribution of asymmetry within the individual spinal cord was very inconsistent. Overall, an asymmetric syrinx only affecting the ipsilateral dorsal quadrant corresponding to the symptomatic side was present in 1/7 cases. In four cases, the majority of the slides (67-86%) revealed asymmetry in the dorsal quadrant corresponding to the ipsilateral symptomatic side, whereas two cases expressed clinical signs contralateral to the side of the spinal cord affected by asymmetric syringomyelia. However, the subsequent evaluation of the dorsal root entry zone demonstrated a clear pattern: In all cases with unilateral clinical signs, the ipsilateral dorsal root entry zone (DREZ) had an abnormal appearance characterised by degeneration and dissection through pia mater in one or more segments, most prevalent in C3 and C4 in 4/7 and 3/7 cases respectively (Fig. 4). In segments with severe, slit-like syringes, the loss of superficial dorsal horn grey matter was evident and seen concomitant with deafferentation characterised by loss of axons and reorganization of first-order axons terminating in deeper grey matter laminae compared to the contralateral dorsal horns (Fig. 4). Other common findings were focal white matter spongiosis and grey matter interstitial oedema. Central canal stenosis was evident in one case, and connective tissue formation entailing aberrant bundles of nerve fibres (axons perpendicular to the longitudinal spinal cord axis) of unknown origin were present in three cases (Fig. 5). No ‘pseudorosettes’, lipid laden ‘foamy’ macrophages, or signs of inflammation were seen. No abnormalities were found in the asymptomatic, syringomyelia-negative controls' cervical spinal cords.

Discussion

The principles of stereological volume quantification imply investigation of a prespecified region of interest. Hence, the present study investigated the spinal cord segments C1-C8 based on previous reports on the predilection site of syringomyelia in the CKCS [14, 18, 38, 39]. A significant grey matter volume loss of the superficial dorsal horn was found in the affected half of the spinal cord in cases with lateralised clinical signs of CNeP compared to the contralateral half. The loss of spinothalamic dorsal horn grey matter is a histomorphological characteristic shared with human syringomyelia patients [23-25]. Despite focal white matter spongiosis, a significant quantifiable volume loss was absent in dorsal, lateral and ventral spinothalamic columns in syringomyelia-affected dogs.

The predominant clinical sign of CNeP was unilateral scratching in 6/7 cases. Itch-induced scratching ca be elicited by i.e. ectoparasites or due to inflammation [40]. Neither ectoparasites nor dermatitis, however, was found in any of the included cases. An itch-specific pathway corresponding to the well-characterised nociceptive pathway has not yet been established. In general, itch is transmitted by pruritoceptive fibres. The fibres synapse with excitatory neurons in laminae I to IV of the dorsal horn [40]; the same area that receive innocuous and nociceptive input from Aβ-, Aδ- and C-fibres [41]. The present study found a clear pattern of ipsilateral changes in the DREZ characterised by deafferentation and reorganization of first-order axons into deeper laminae in cases with lateralised clinical signs; histopathological changes that has not previously been reported in CKCS with syringomyelia-related CNeP. Our findings are in accordance with a study by Milhorat *et al.*, who published a similar description of dissecting syringomyelia through pia mater in the DREZ in 5/12 human patients with Chiari-1 malformation related syringomyelia [25]. Four of these presented with allodynia or hyperalgesia in a dermatomal pattern that corresponded to the DREZ-pathology. In a study of pain-related somatosensory laser-evoked potentials in eight human patients with syringomyelia-related neuropathic pain and mixed loss of sensation, Kakigi *et al.* found an impaired dorsal horn function in 6/8 patients and intact function of the ascending fibres in 7/8 patients [42]. In contrary, an experimental rodent model of excitotoxic spinal cord injury, Yeziersky *et al.* reported behavioural indication of neuropathic pain characterised by excessive grooming and lowered thresholds to mechanical stimulation with monofilaments despite sparring of superficial dorsal horn grey matter [43]. Whether the present

DREZ-pathology in the CKCS with syringomyelia-related CNeP is consistent with the experimental evidence of neuropathic itch and mechanical threshold alterations or a consequence of impaired dorsal horn inhibition after axonal injury (i.e. spinothalamic neuronal loss or a consequence of primary deafferentation with secondary trans-synaptic neuronal loss) remains unknown.

The primary aim of the present study was not to elucidate the underlying mechanism of syrinx formation. However, we identified a general pattern of independency between the central canal and the emerging syrinx formation at the cranial origin of syrinx. The central canal dilation was seen with a concomitant incipient cavity formation in the dorsal quadrant's grey matter. The authors hypothesise that changes in cerebrospinal fluid flow dynamics due to Chiari-like malformation in the CKCS may affect the spinal cord compartments, tissues and homeostasis differently. A possible explanation of the incipient slit-like cavity in the dorsal quadrant's grey matter could be the increased susceptibility to hypoxia of GABAergic neurons [44-46].

The present study protocol strived to reduce the variation in pre-processing tissue handling and post-mortem changes as much as possible. Compared to a previous histopathological case-series in syringomyelia-affected CKCS [22], the lesion classification was less biased by post-mortem artefacts. In addition, the strict inclusion of clinically well-characterised symptomatic cases was undertaken to increase the likelihood of identifying relevant pathological findings as an explanation of clinical signs. However, the limited sample size and the variation between the included dogs introduced a number of limitations. The variation in age and size between the four controls dogs resulted in a broad reference range for comparisons with cases. The older a control dog was, the greater tendency to central canal dilation. This reflects the great inconsistency in the definition and classification of central canal dilation, hydro- and syringomyelia both at the histological level as well as on MRI and in particular the challenges met in the assessment of the MRI findings' clinical significance [8, 47]. Some tissue-sections from one case and the youngest control dog were stained free-floating. This process may have introduced bias in the area-estimations due to processing artefacts. In general, tissue processing before staining results in length, area and volume shrinkage of approximately 15%, 30% and 50% [48, 49]. The measures in the present study will therefore underestimate the diameters, areas and volumes accordingly.

Future studies could undertake systematic random sampling of the dorsal roots and ganglions to investigate, if the loss of dorsal horn grey matter is a consequence of reactive deafferentation or a result of primary deafferentation with secondary degeneration. Quantitative microscopy of spinal cords immunohistochemically labelled for microglia cells and proinflammatory cytokines (IL-1, IL-2, IL-6, IL-7, and TNF) could determine if microglial activation and cytokine-induced long-term potentiation of excitatory dorsal horn projection neurons is involved in CNeP generation [50-53]. Furthermore, caspase3 or TUNEL should be used evaluate, if neuronal apoptosis is involved in grey matter loss [54]. Quantitative immuno-fluorescence microscopy of specific sub-populations of dorsal horn GABAergic and glycinergic interneurons would reveal, if excitotoxic neuronal damage or a phenotypic shift in GABAergic and glycinergic neurons from inhibition to excitation is present in syringomyelia-affected dogs [55, 56].

In conclusion, an association between lateralised clinical signs and a grey matter volume loss of the superficial dorsal horn was established in CKCS with syringomyelia. The unilateral slit-like syrinx formation in dogs with lateralised clinical signs caused a degeneration of the dorsal root entry zone with dissection through pia mater. The findings offer a structural explanation of the CNeP symptoms in CKCS with syringomyelia. Moreover, the high prevalence of syringomyelia in the CKCS enable investigations of the possible biochemical and functional consequences of the spontaneous CNS lesions and their relevance to clinical signs of CNeP. Systematic sampling of the dorsal roots and ganglions should be undertaken to elucidate, if the loss of dorsal horn grey matter is a consequence of reactive deafferentation or a result of primary deafferentation with secondary trans-synaptic degeneration. Establishing the causal relation between syringomyelia and CNeP in the CKCS potentially offers an appropriate analogy model to human syringomyelia-related central neuropathic pain. Future in-depth neurohistopathological quantification and characterisation of this spontaneous model potentially enables the identification of new drug targets and consequently the design of novel therapies.

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Table 1. Demographics of included dogs.

Demographics of the 12 Cavalier King Charles Spaniels included in the study. Continuous variables are reported as median (range). Categorical variables are reported as number.

	Controls n=4	Cases n=8
Age (years)	5.1 (0.8 - 8.8)	5.2 (2.5 - 12.4)
Sex (F / FN / M / MN)	4 / 0 / 0 / 0	4 / 1 / 2 / 1
Bodyweight (kg)	7.6 (6.7 - 8.2)	10.2 (8.7 - 13)◊
Age at MRI (years)	2⋆	3.7 (2.2 - 7.3)
MRI characteristics		
Chiari-like malformation (yes / no)	1	8 / 0
Syrinx : spinal cord ratio +	0	0.6 (0.44 - 0.75)
OME (no / bilateral / unilateral)	1 / 0 / 0	4 / 2 / 2
Time from MRI to euthanasia (years)	0.6	1.1 (0 - 7.3)
NeP score at the day of euthanasia	0	2.1 (0.9 - 2.9)
Clinical phenotype characteristics		
NRS scratch	0	4 (0 - 9)
Uni- or bilateral scratching (UL / UR / B)	-	4 / 3 / 1
Scratching localisation (ears / neck / shoulder / axilla / abomen)	-	5 / 5 / 6 / 1 / 2
NRS pain	0	2.5 (1 -6)
Current analgetic treatment regimen		
Carprofen 37.5 mg SID / firocoxib 57 mg PO SID	-	1 / 1
Prednisolone 5 mg SID / Methylprednisolone 4 mg PO SID	-	1 / 1
Tramadol 25 mg PO TID	-	1
Gabapentin 150 /200 / 300 / 400 mg PO TID	-	1 / 1 / 2 / 1
Pregabalin 150 / 300 mg PO BID	-	1 / 1

F, female; **FN**, female neutered; **M**, male; **MN**, male neutered. **NRS**, numeric rating scale (0 - 10). **UL**, unilateral left; **UR**, unilateral right; **B**, bilateral. **PO**, per os. **SID**, q24h; **BID**, q12h; **TID**, q8h. ◊ The bodyweight was significantly different between controls and cases (*P*=0.0046). ⋆ One of the four controls had MRI of the neurocranium and cervical spinal cord as a part of the clinical work-up due to severe undesirable behaviours. MRI was not indicated in the work-up of the additional control dogs due to absence of neurological symptoms. + The syrinx / spinal cord ratio is the ratio between the diameter of the syrinx and spinal cord respectively measured, where the syrinx was widest.

Table 2 Descriptives: autopsy details and region of interest.

Data on autopsy times and measurements (length and weight) of the spinal cord segments C1-C8 included in the study. Data are reported as mean (range). None of the reported parameters were significantly different between cases and controls.

	Controls n=4	**Cases** n=8
Time from euthanasia to autopsy (minutes)	33 (10 - 130)	13 (5 - 55)
Autopsy duration (minutes)	58 (45 - 93)	68 (65 - 80)
Time from euthanasia to tissue in fixative (minutes)	105 (75 - 175)	87 (75 - 125)
Spinal cord segments C1-C8 length (cm)	10.2 (9.7 - 10.5)	11 (9.4 - 12.5)
Spinal cord segments C1-C8 weight (g)	6 (5.3 - 6.3)	6.7 (5.1 - 8.3)

Table 3 Total volume estimates.

Specific volume estimates of the total mean volumes of the spinal cord, grey matter, white matter and central canal or tissue loss due to syringomyelia in controls (n=4) and cases (n=8). In addition, total sub-volumes of the predefined anatomical regions corresponding to the spinothalamic functional units: the dorsal horn laminae I-III as a representation of the 2nd order nociceptive neurons of the ascending pathway; the white matter's dorsal funiculi representing the spinothalamic dorsal column, the lateral funiculi representing the lateral spinothalamic columns and the ventral funiculi representing the ventral area of spinothalamic fibre decussation. Comparisons of the volume-estimates between cases and controls revealed a significant difference in the total volume of the central canal (*P*=0.04). A subsequent computation of the total volumes of the grey and white matter, and relevant spinothalamic functional units of the affected and non-affected half of the spinal cord in cases (n=7) with lateralised symptoms was undertaken. A paired comparison of the total volumes revealed a significant volume loss of the dorsal horn laminae I-III on the affected side to which the symptoms were ascribed (*P*=0.034). Additional comparisons were insignificant.

				CASES with lateralised symotoms (n=7)		
MEAN (range) VOLUME (μm3)	CONTROLS (n=4)	CASES (n=8)	*P*	NON-AFFECTED SIDE	AFFECTED SIDE	*P*
SPINAL CORD TOTAL VOLUME	2605 (1974 - 3601)	2860 (1882 - 3943)	0.56			
CENTRAL CANAL / SYRINX VOLUME	13 (6 - 26)	188 (49 - 461)	*0.04*			
GREY MATTER TOTAL VOLUME	569 (405 - 850)	539 (345 - 807)	0.78	302 (175 - 405)	262 (170 - 402)	0.19
LAMINAE I-III	88 (55 - 144)	76 (55 - 102)	0.47	42 (27 - 54)	34 (20 - 48)	*0.034*
LAMINAE IV-X	481 (335 - 707)	460 (290 - 713)	0.82	231 (148 - 354)	255 (143 - 359)	0.11
WHITE MATTER TOTAL VOLUME	2193 (1609 - 2960)	2334 (1580 - 3203)	0.7	1111 (791 - 1498)	1099 (789 - 1528)	0.48
DORSAL FUNICULI	456 (324 - 646)	430 (261 - 607)	0.73	215 (132 - 301)	204 (130 - 305)	0.13
LATERAL FUNICULI	1642 (1218 - 2210)	1804 (1257 - 2575)	0.57	848 (629 - 1134)	846 (627 - 1161)	0.91
VENTROMEDIAL FUNICULI	95 (67 - 110)	99 (61 - 145)	0.82	48 (30 - 73)	48 (31 - 72)	0.76

Figure 1

Region of interest The spinal cord segments C1-C8 were sampled for stereological total volume quantifications and histopathological evaluation from four asymptomatic controls and eight symptomatic, syringomyelia-positive cases. The landmarks used for transection of the exposed spinal cord was the most cranial and caudal emergences of the paired dorsal spinal nerves I and VIII respectively. The length of the depicted spinal cord segments C1-C8 from an asymptomatic, 8.8-year-old intact female Cavalier King Charles spaniel is 10 cm.

Figure 2

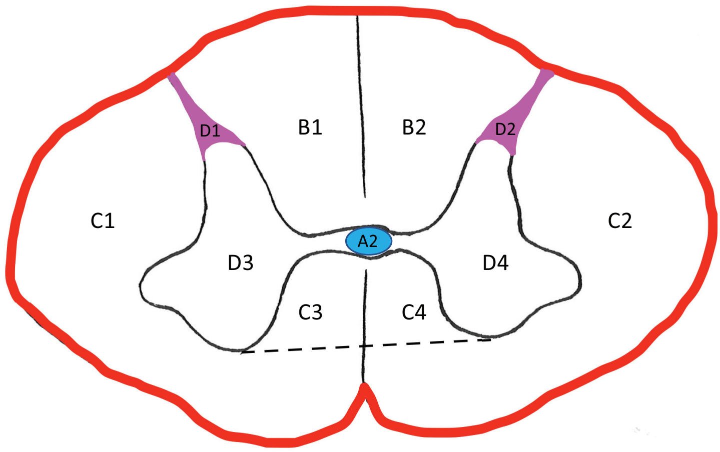
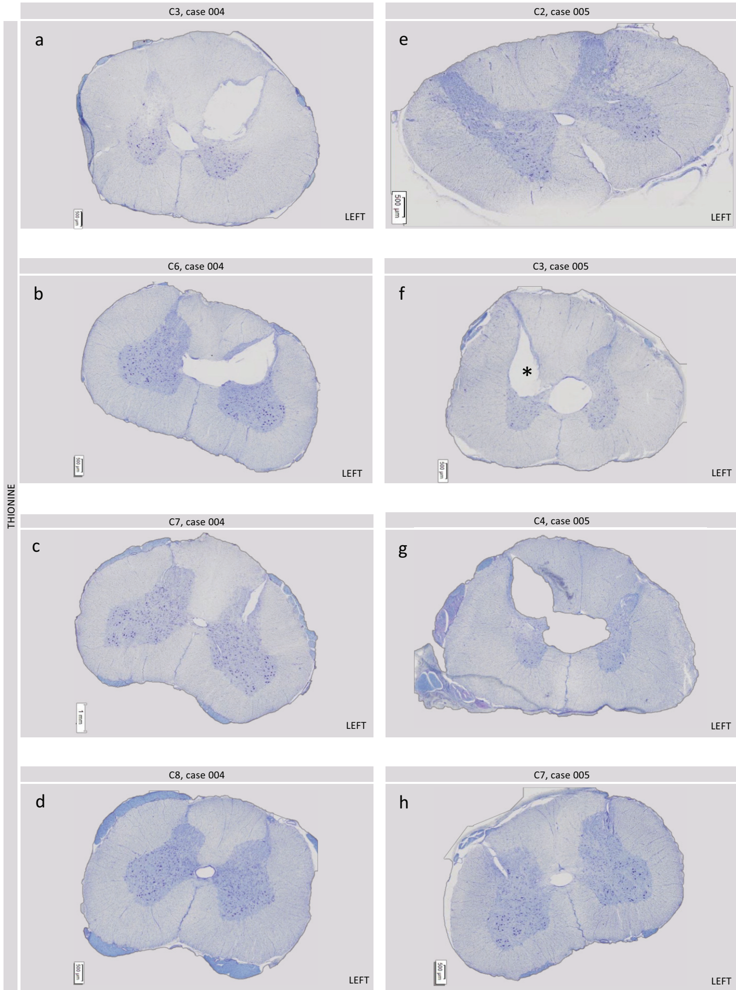


Illustration of the specific anatomical spinal cord entities included in volume estimation

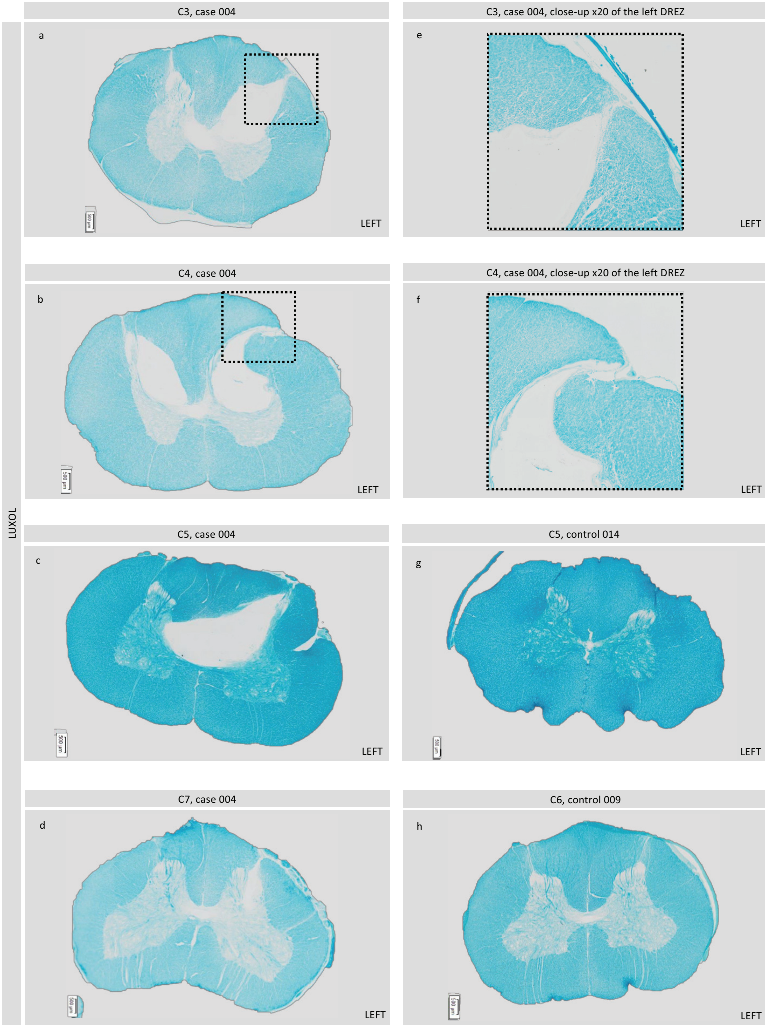
Serial transverse 10 µm sections immunohistochemically stained with the neurofilament triplet H protein-specific primary antibody, SMI-32 were used to delineate the following predefined anatomical regions corresponding to the spinothalamic functional units: **D1** and **D2**, the left and right dorsal horn laminae I-III as a representation of the 2nd order nociceptive neurons of the ascending pathway; the white matter's dorsal funiculi (**B1** and **B2**), lateral funiculi (**C1** and **C2**) and ventral funiculi (**C3** and **C4**) as representatives of the dorsal and lateral spinothalamic columns and the ventral area of spinothalamic fibre decussation. The areas of these predefined spinothalamic functional units were estimated in addition to the total area of the cervical spinal cord (demarked in **red**), the remaining grey matter (**D3** and **D4**) and the central canal (**A2**) or, in case of syringomyelia, the area of tissue loss.

Figure 3



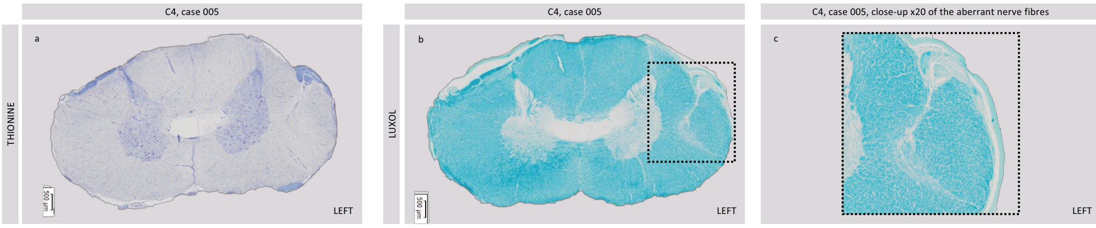
Cranial and caudal poles of the syringes in two cases Photomicrographs of 30 µm transverse sections stained with thionine. Pictures to the left demonstrate the cranial and caudal pole of the syrinx in a 2.5-year-old female case with unilateral scratching on the left side of the neck. Pictures to the right demonstrate the cranial and caudal pole of the syrinx in a 5-year-old male case with unilateral scratching on the right side of the neck. The cavity formation was independent on the central canal in the cranial pole of the syrinx in both cases (**a** and **f**). The fusion-pattern between the central canal and the syrinx seen in 5/8 cases is demonstrated in (**b** and **g**). In segments caudal to the caudal pole of the syrinx, a distinct central canal was seen in 6/8 cases (**d** and **h**). ***** Example of a slit-like syrinx shape

Figure 4



Dorsal root entry zone pathology Photomicrographs of 30 µm transverse sections stained with luxol. In 7/7 cases (case 004 is shown here to exemplify) with unilateral symptoms, the ipsilateral dorsal root entry zone had an abnormal appearance characterised by degeneration and dissection through pia mater (**a** - **d** and close-up **e** and **f**). The significant volume loss of dorsal horn grey matter was accompanied by deafferentation (loss of axons) and reorganisation of the first-order axons, that subjectively were assessed to terminate in deeper laminae compared to the contralateral dorsal horn and controls (**g** and **h**).

Figure 5



Aberrant nerve fibres Photomicrographs of 30 µm transverse sections stained with **a**) thionine and luxol (**b** and **c**). The sections display aberrant nerve fibres. The fibres penetrate the pia mater and enters the spinal cord lateral funiculus perpendicular to the neuraxis.

Paper IV

Pregabalin alleviates clinical signs consistent with syringomyelia-related central neuropathic pain in Cavalier King Charles Spaniels – a randomised controlled trial

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Pregabalin alleviates clinical signs of syringomyelia-related central neuropathic pain in Cavalier King Charles Spaniels – a randomised controlled trial

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Running head Pregabalin in canine central neuropathic pain

Keywords Analgesia, canine chronic pain, Chiari-like malformation, clinical pharmacology, neuralgia, spinal cord disease

Authors' contributions
MST: principal investigator, study design, data acquisition, management and interpretation, statistical analysis and manuscript preparation;
LTS: data interpretation, statistical analysis and critical revision of the manuscript;
FM: data acquisition and manuscript review;
MB: study design and manuscript review;
OJB: study design, data interpretation and critical revision of the manuscript.

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Abstract

Objective Cavalier King Charles Spaniels with syringomyelia express clinical signs of central neuropathic pain. Predominant clinical signs of pain are scratching and paroxysmal pain manifestations with vocalisation. No evidence-based treatment for canine central neuropathic pain exists. Since pregabalin alleviates human neuropathic pain we aimed to investigate pregabalin's effect on canine syringomyelia-related central neuropathic pain.

Study design An authority-approved, randomised, crossover superiority trial.

Animals Twelve client-owned dogs with magnetic resonance imaging-confirmed syringomyelia and clinical signs of central neuropathic pain, aged between 1.1 and 7.4 years, weighing between 8.2 and 10.8 kg.

Methods Randomisation of dogs to either pregabalin 150 mg or placebo for 25 days and 48-h washout before crossover to the alternate phase of 25 days duration. The primary outcome was defined as number of scratching events during ten minutes of physical activity. The treatment effect was estimated using a generalised estimation equations model. Benefit-risk assessment of treatment was obtained through observation of side effects, adverse events and owner satisfaction.

Results The treatment effect estimate was an 84 % (CI95 = 75%, 89%) reduction in mean number of scratching events relative to baseline when compared to placebo (P = <0.0001). The administered dose range (13 - 19 mg/kg q 12 h) was well tolerated in all but one dog (withdrawn seven days after crossover to pregabalin due to ataxia). The most prevalent side effects were increased appetite in 9/12 dogs and ataxia in 9/12 dogs. Improved quality of life was reported in all dogs. No adverse events were seen. No dogs needed rescue analgesia during the trial.

Conclusion and clinical relevance Pregabalin is superior to placebo in the reduction of clinical signs of syringomyelia-related central neuropathic in dogs. A dose range of 13 - 19 mg/kg q 12 h is safe to use and improves the dogs' quality of life.

Introduction

Cavalier King Charles Spaniels (CKCS) with Chiari-like malformation and syringomyelia (SM) express clinical signs of central neuropathic pain (CNeP) (Rusbridge et al. 2007; Rutherford et al. 2012; Hechler & Moore 2018). The clinical presentation of the canine SM phenotype is very diverse. Typical manifestations of discomfort and pain reported by the owners are spontaneous and evoked scratching, phantom scratching, paroxysmal pain manifestations with vocalisation and different aberrant behaviours e.g. nightwandering, hiding, avoidance of touch and grooming and reluctance to wear a collar or harness (Rusbridge et al. 2000; Sanchis-Mora et al. 2016; Sparks et al. 2018).

To date no evidence-based recommendations for the treatment of clinical signs of SM-related CNeP has been published. The structural gamma-aminobutyric acid (GABA) analogue pregabalin (PGN) has been found effective for human neuropathic pain patients in several randomised controlled trials and is classified as a first-line analgesic (Siddall et al. 2006; Vranken et al. 2008; Cardenas et al. 2013; Finnerup et al. 2015). Accordingly, the primary aim of the study was to assess the efficacy and benefit-risk profile of PGN in dogs with clinical signs of CNeP associated with SM. We hypothesise that the analgesic efficacy of PGN is superior to placebo to reduce clinical signs of SM-related CNeP.

Materials and methods

The trial was conducted at the University Hospital for Companion Animals (UHCA). The study was approved by the Danish Medicines Agency (11th of April 2017, file number 2017020400) and the local Ethics and Administration Committee (20th of February 2017, file no. 2017-4). Informed content was obtained from all owners.

Study population

Eligible client-owned, purebred CKCS older than one year of age dogs should weigh ≥ 8 and ≤ 12 kg, express clinical signs of SM-related CNeP with MRI confirmed SM. Clinical signs of SM-related CNeP was defined as uni- or bilateral spontaneous scratching directed at the shoulder area. SM was defined as a fluid filled cavity in the spinal cord parenchyma with a diameter ≥ 2 mm on T1-weighted images. Owners should be willing to administer an ectoparasite prophylaxis (e.g. fipronil, imidacloprid/permethrin or fluralaner) before inclusion. Pregnant or lactating dogs and dogs treated with any kind of analgesics 48 hours prior to inclusion were not eligible.

Pre-inclusion visits were undertaken by the principal investigator (PI [MAST]) and a dedicated research veterinary technician. The questionnaire published by Rutherford *et al*. (Rutherford et al. 2012) including questions regarding the dog's general health status, medical history, clinical signs, behaviour and quality of life (QOL) was used to confirm eligibility and to establish the individual dog's baseline neuropathic pain score. The dogs were walked on a predefined route for ten minutes to confirm that they expressed clinical signs of SM-related CNeP. The walk was video-documented to enable retrospective re-assessment of the dogs' scratching profiles and quantification of scratching events. The dogs underwent a clinical and neurological examination, otoscopy, ear swab cytology, urine- and blood analysis including CBC, biochemical and thyroid profile before magnetic resonance imaging (MRI) of the neurocranium and cervical spinal cord parenchyma as previously described by Thoefner *et al*. (Thoefner et al. 2019) to rule out other causes of scratching than SM.

Study design and blinding procedure

This superiority trial was designed as a two-treatment two-period crossover trial with random assignment of elective dogs to treatment arm A with treatment sequence 'PGN - placebo' or treatment arm B with treatment sequence 'placebo - PGN' (Figure 1). The allocation ratio was 1:1. A simple randomisation code was used. To enrol a dog, the PI asked the owner to draw a random number from a non-transparent envelope. The random number corresponded to a unique trial code on the randomisation list generated by an online randomisation tool (www.randomization.com). The list was provided in a sealed envelope by the pharmacist who manufactured, packed and labelled the investigational agent and placebo capsules.

Gelatine capsules of identical appearance containing either placebo or PGN (Lyrica; Pfizer, NY, USA) were provided in containers of identical appearance. Containers could only be distinguished by the label, which stated the unique trial code and 'treatment period one' or 'treatment period two'. Thus, randomisation to treatment arm A or B was blinded to the PI and the owner. To eliminate any biased expectation of effect, the owners were not informed of the name of the investigational agent. The trial was continuously followed by an external monitor. The PI, owners, monitor and statistician were blinded to the treatment sequence allocation. The last-patient-last-visit defined the end-of-trial. The dataset was completed and locked, and the code was not broken until data analysis were finalised.

Intervention

Patients randomised to treatment arm A received PGN PO, initially 150 mg q 24 hours for two days increasing to a targeted maintenance dose of 150 mg q 12 hours for 21 days, followed by a tapering phase of 150 mg q 24 hours for two days. The washout period (48 hours) was followed by crossover to the placebo-treatment period: one capsule q 24 hours for two days, one capsule q 12 h for 21 days followed by 1 capsule q 24 h. The dogs randomised to treatment arm B were subjected to 25 days of placebo-treatment, two days washout followed by 25 days of PGN-administration as described for dogs in treatment arm A (Figure 1). No concomitant analgesic treatment was allowed during the trial. If the owner or PI assessed a failure to respond that affected the dog's QOL, the dog was withdrawn from the study and rescue analgesia was initiated.

Outcome measures

The outcomes were assessed five times in total: at the pre-inclusion visit where baseline data were collected, and at four follow-up visits (two visits in each treatment period, day 7, 21, 34 and 48 $\pm$ 2 days).

The primary outcome 'number of scratching events during ten minutes of continuous, physical activity' was documented in standardised, video-series of ten minutes duration and quantified by counting scratching episodes.

Secondary outcome measures for scratching were assessed by the owner by means of the 11-point numerical rating scale, SCR/NRS where 0=no scratching and 10=worst scratching imaginable. Subsequently, the owner was presented to a modified Faces Pain Scale designed for pain assessment in children with five faces, a colour-intensity scale beneath the faces and a horizontal sliding indicator (Hicks et al. 2001). The owners were asked to place the sliding indicator on the colour or face that corresponded to their assessment of their dogs' scratching intensity, SCR/VAS during the last 24 hours. On the back side of the modified Faces Pain Scale was a pre-printed 0-100 mm VAS scale, and the SCR/VAS score corresponding to the owner's placement of the horizontal sliding indicator on the front side was read by the PI.

The dog's pain / discomfort intensity was also rated on the numeric rating scale. Assessment of PAIN/NRS ranged from 0=no pain/discomfort to 10=worst pain/discomfort imaginable. The above described modified Faces Pain Scale was used for the owner's rating of pain / discomfort, PAIN/VAS. Finally, the owner was asked to rate the dog's QOL as 'could not be better', 'good', 'fairly good', 'neither good nor bad', 'fairly poor', 'poor', 'could not be worse' or 'do not know'.

The PI rated the dog's scratching intensity and pain / discomfort respectively by means of the same NRS and VAS-scales as the owner after each visit. In addition, the PI assessed the dog's subjective QOL based on the history, clinical findings and overall subjective impression of clinical efficacy of the initiated treatment on number of scratching events and stress level.

The owners were provided with a diary at enrolment to log deviations of medicine administration deviated from the predefined time, the daily scratching intensity, SCR/NRS and to document any side effects and other observations during the trial period. The dogs' body weight was continuously recorded during follow-up visits. Additionally, the owners were asked a series of closed and semi-open questions at each follow-up visit to monitor side effects and adverse events. The questions addressed activity level, appetite, aggression, ataxia, behavioral changes, fecal score, food intake, somnolence, vomiting, water intake and details on their dog's scratching phenotype.

To account for end-of-trial owner-compliance, the remaining number of capsules were counted at each follow-up visit. The proportion of capsules given relative to the expected number of capsules given at each follow-up visit was used to categorise owners as 'satisfactory adherent' (≥ 90% capsules administered correctly) or 'suboptimal administrator' (≥ 80% and ≤ 90% capsules administered correctly) (Pullar et al. 1989; Dodd et al. 2012). At the last follow-up visit owners were asked for a final preference statement (treatment period one or two).

Statistical analysis

Data were analysed with SAS Studio 3.71 (SAS Institute Inc.; NC, USA). $P < 0.05$ was considered significant. Descriptive statistics for categorial variables are reported by frequencies and proportions and for continuous data as medians and ranges except for the primary outcome 'number of scratching events' which is reported as mean and range. Since 'number of scratching events' is a count variable, a negative Binomial distribution was used to model it, allowing for overdispersion (as compared to a Poisson distribution). The effect of treatment (PGN or placebo), period, follow-up visit number and potential carryover was modelled using a log-link (i.e. multiplicative effects), and a generalised estimating equation was applied to account for the correlation of scratching events over time for the same dog (Zeger & Liang 1986). The effect estimates are given as ratios, e.g. of PGN vs. placebo. Treatment effect on owner and PI-assessed secondary outcomes SCR/NRS, SCR/VAS, PAIN/ NRS and PAIN/VAS was modelled by means of a general linear mixed model as was the effect on body weight after log-transformation (Liang & Zeger 1986). Cross-tabulations were made to assess the agreement between owners and the PI with regard to the dogs' QOL. Data were included in the analysis when dogs had participated in at least three of four consecutive follow-up visits. If owners answered a question with 'do not know', data were excluded from analysis.

Results

81 potential cases were consecutively assessed for eligibility between March 2017 and June 2018 (see trial profile, Figure 2). Seventeen eligible dogs underwent pre-inclusion visits. Four of these were excluded (due to severe proprioceptive deficits, aberrant, obsessive compulsive behaviour, lack of owner compliance and one dog only scratched during oestrus). The thirteen remaining dogs were consecutively included in the study from April 2017 to June 2018 and randomised to treatment arm A (n=5) or B (n=8) after baseline data collection. The last follow-up visit took place in July 2018. Eleven dogs completed the trial. One dog was excluded after follow-up I due to non-compliance to the protocol and is therefore not included in the reported results, and one dog was withdrawn by the owner after follow-up III due to ataxia and somnolence. No dogs needed rescue analgesics during the trial.

At baseline, the 12 participants (six females, one neutered female, four males and one castrated male) had a median age of 3.6 years (1.1 - 7.4), a median bodyweight of 8.7 kg (8.2 - 10.8) and a median NeP-score of 1.1 (0.7 - 2.6). The overall mean number of scratching events during ten minutes of continuous physical activity was 9.1 (2 - 27). Additional information including MRI findings, owner and PI-assessed baseline characteristics of the 12 dogs are presented in Table 1.

Treatment effect

The treatment effect of PGN on the mean number of scratching events was estimated to be a factor 0.16 (CI_{95} = 0.11, 0.25) corresponding to an 84% reduction from baseline in the mean number of scratching events during ten minutes of continuous physical activity when compared to placebo (*P* = <0.0001, see Figure 3). No significant effects of neither placebo, period, follow-up visit number nor carryover was seen.

Efficacy estimates of PGN on secondary outcomes showed a significant reduction from baseline in owner-reported mean scratching and pain intensities on both the numeric rating scale (SCR/NRS of -2.7 (*P* = 0.003)) and the modified Faces Pain Scale (SCR/VAS of -33 (*P* > 0.0001) and PAIN/VAS of -24 (*P*=0.01)). The same significant effect of PGN on PI-assessed secondary outcomes was seen, since the mean SCR/NRS was reduced by -2.7 (*P*=0.0016), SCR/VAS by -29 (*P*=0.001), PAIN/NRS by -4.1 (*P*<0.0001) and PAIN/VAS by -44 (*P*<0.0001). The only exception, where no difference was seen in effect between PGN and placebo, was on owner-assessed PAIN/NRS (*P*=0.056). The efficacy estimates' confidence limits are reported in Table 2.

Additional information reported by the owners

According to the owners' diary records, the clinical effect of PGN was observable 48-72 hours after the PGN administration was initiated. Hereafter, the dogs were not scratching for consecutive days at home during the PGN-treatment period. The scratching profile changed during treatment with PGN: the phantom scratch stopped in seven of eight dogs and vocalisation when scratching ceased in five dogs (100%).

External factors that affected the owners' daily SCR/NRS ratings were: wearing a harness or a collar in six of the included dogs, flea infestation, which was promptly treated in two of the included cases in arm B during treatment period two, and increased scratching intensities during oestrus was reported in two females during the trial. Three dogs were initially reluctant to take medications and four owners found it difficult to fit the 12-hours dosing intervals into their daily routines.

Side effects and adverse events

The prevalence of owner-reported side effects and adverse events continuously registered at each follow-up visit is presented in Table 3. The most prevalent side effects following PGN administration were increased appetite in 9/12 dogs and transient ataxia which resolved within one to ten days in

9/12 dogs. The body weight of included dogs ranged from 8.0 kg to 11.4 kg during the trial. The resulting administered dose range was 13 - 19 mg/kg q 12 h. Overall, this dose of PGN was well tolerated in all but the one dog, that was withdrawn by the owner due to ataxia as previously described. Despite the reported increase in appetite and a clinical overt weight gain in four dogs, the overall increase in the dogs' mean bodyweight of 2.1 % (CI_{95} = 0.4%, 4.6%) during PGN treatment was non-significant ($P < 0.099$). The weight gain was more evident in four ad-lib fed dogs compared to the other seven dogs whose owners were more attentive to restricted feeding. No adverse events were seen during the trial.

Quality of life

The owner-assessed QOL improved in 38% of dogs allocated to arm A (PGN - placebo) and in 36% of the cases allocated to arm B (placebo - PGN) during treatment with PGN relative to baseline assessment. During the placebo-period, the owner-assessed QOL improved in 25% of the dogs in arm A whereas none of the owners reported improved QOL during placebo treatment for dogs allocated to arm B. A cross-tabulated comparison of the overall agreement between the owner and PI-assessed QOL revealed agreement in 30/58 assessments. In 21 of the remaining 28 cases, the owner evaluated a better QOL, which was significantly more often than the PI (P=0.016).

Compliance

The overall owner compliance (number of capsules administered relative to the number of capsules expected to be administered) was 98% (range 93% - 105%). One owner had forgotten to up-titrate during treatment period two. Consequently, the duration of treatment period two was only 24 days, which results in the "over-compliance" of 105% (46 capsules administered in 44 days). All owners in treatment arm B and two owners in treatment arm A (50%) were categorised as 'satisfactory adherent' (> 90% capsules administered correctly) at all 4 follow-up visits. Two treatment arm A-owners (50%), were categorised as 'suboptimal administrators' (≥ 80% but ≤ 90% capsules administered correctly) during the placebo-period.

Efficacy of blinding and final preference statement

To assess the efficacy of blinding, owners were asked for a final preference statement at the end-of-trial visit. Despite blinding to treatment allocation and the reported side effects, all 12 owners preferred the period in which their dog had received PGN over the placebo-period.

Discussion

The present randomised controlled trial provides convincing evidence that PGN significantly alleviates clinical signs of SM-related CNeP in CKCS. The estimated treatment effect of PGN was a reduction of 84% in the mean number of scratching events relative to baseline. However, the relatively wide confidence interval of 75% - 89% indicates some clinical variation in the response between dogs. In addition, PGN significantly reduced the owner- and PI-assessed NRS and VAS scratching- and VAS pain intensities and improved QOL in all the included dogs. To the authors' knowledge this is the first evidence-based treatment recommendation to reduce clinical signs of SM-related CNeP. The analgesic effect of another human-labelled GABA-analogue, gabapentin has been found non-significant in dogs with postoperative and bone cancer pain (Wagner et al. 2010; Aghighi et al. 2012; Crociolli et al. 2015; Monteiro et al. 2018). A single-blinded trial of gabapentin as an add-on to carprofen in CKCS with clinical signs of SM reported a significant improvement in QOL as an indirect indicator of pain alleviation compared to baseline (Plessas et al. 2015). The authors speculated, if insufficient dose rationales can explain the non-significant effect in the previous studies rather than a veritable lack of effect.

The primary outcome definition 'number of scratching events during 10 minutes of continuous observation' was based on previous reports on the most prevalent clinical signs in CKCS with SM (Rusbridge et al. 2000; Rusbridge et al. 2007; Sanchis-Mora et al. 2016; Sparks et al. 2018). As scratching is a common, daily observed behaviour in dogs, the authors acknowledge that not all types of scratching in CKCS can be ascribed to the SM-related parenchymal spinal cord changes alone. Consequently, dogs were excluded if their scratch was solely directed at the ears or face. This very strict inclusion of cases served to avoid the potential confounding effects of Chiari-like malformation and otitis media with effusion as primary causes of scratching. Overall, a great variation in the primary outcome within and between dogs was seen during follow-up visits. According to the owners, the dogs' scratching was more evident at the day of follow-up visits than for several days prior to the follow-up visits. External stressors introduced in the home environment as well as wearing a collar or harness has previously been reported as a cause of scratching intensification (Rusbridge et al. 2000; Rutherford et al. 2012). Transportation, often by car, entering the hospital facility and wearing a collar or harness during video recordings, flea infestation and oestrus were all confounding factors that most likely contributed to this variation and the resulting relatively broad confidence interval of the primary outcome. Ectoparasite prophylaxis was initiated in all dogs at inclusion. Repeated prophylactic treatment when indicated was not incorporated in the protocol which explain the unfortunate flea infestation towards the end of trial period two.

The trial was designed to mimic the clinical setting to document the real-life effect of PGN. For that reason, we deselected a run-in period. Although the overall owner compliance was very good, variations within and between owners and dogs that could affect the primary outcome measure were seen: initial reluctance among dogs to accept PO administration of PGN and fluctuations in the owners' daily routines not always compatible with the 12 h dosing interval. The above mentioned physiological, environmental and practical factors are difficult to control for in a clinical trial but the nature of the crossover design reduces the impact on the overall conclusions.

The benefit-risk profile assessment indicates, that PGN is safe and well tolerated in the applied regimen of 13 - 19 mg/kg q 12 h. The most prevalent entailed side effects were an increase in appetite, a resultant increase in body weight in ad-lib fed dogs and transient ataxia of less than ten days duration. The latter was subjectively described by the owners as acceptable and resolved within one to ten days. Only one owner assessed that side effects exceeded benefits in the reduction of clinical signs and withdrew the dog from the study. Based on the reported possible side effects listed in PGNs' summary of product characteristics (Lyrica SPC)(Lyrica SPC), the side effects reported in the present study were to be expected. No placebo effects could be identified during the data-analysis. Interestingly, three owners reported potential nocebo-effects during the placebo-period: polyphagia in two dogs and ataxia in one dog, that could not be confirmed on clinical examination. A major advantage of PGN treatment which was described by all owners was an improved QOL.

In conclusion, this is the first independent, authority-approved controlled clinical trial that reports a significant reduction in SM related clinical signs of CNeP and improved QOL in client-owned CKCS. Based on our results, we provide an effective and safe evidence-based treatment recommendation of PGN 13 - 19 mg/kg q 12 h. Furthermore, the present study enables future randomised, controlled trials using an active and safe comparator rather than placebo in dogs with

SM related clinical signs of CNeP. This short-term study proves treatment effect, but to assess if chronic administration of PGN in dogs is safe, a longitudinal cohort study with continuous monitoring of effective plasma PGN concentration and potential adverse effects on organ systems is needed. Finally, before treatment is initiated in the clinical setting, an important distinction between statistical significance and the clinically relevant treatment efficacy must be communicated to owners. It must be emphasised that monotherapy with PGN will alleviate the dog's clinical signs significantly without being a remedy for the underlying, progressive spinal pathology that causes the clinical signs.

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Table 1 Baseline characteristics.

Baseline characteristics of the 12 Cavalier King Charles Spaniels included in the trial data analysis. Continuous variables are reported as median (range) except for the primary outcome '**number of scratching events**,' that is reported as mean (range). Categorical variables are reported as number.

	Before randomisation (n = 12)	
Age (years)	3.6 (1.1 - 7.4)	
Sex (F / FN / M / MN)	6 / 1 / 4 / 1	
Bodyweight (kg)	8.7 (8.2 - 10.8)	
NeP score	1.1 (0.7 - 2.6)	
Number of scratching events	9.1 (2 - 27)	
MRI characteristics		
Chiari-like malformation (yes / no)	13 / 0	
Syrinx : spinal cord ratio	0.59 (0.29 - 0.7)	
OME (no / bilateral / unilateral)	6 / 4 / 2	
Scratching intensity	**Owner-assessed**	**PI-assessed**
NRS, 0-10	4 (1 - 7)	6 (1-8)
VAS, 0-100 mm	42 (28 - 65)	62 (12 - 79)
Pain intensity		
NRS, 0-10	2.7 (0 - 5)	4.5 (2 -8)
VAS, 0-100 mm	35 (0 - 50)	50 (22 - 79)
Quality of life		
Could not be better / good / fairly good / neither good, nor bad / bad	3 / 4 / 4 / 0 / 0*	0 / 4 / 5 / 2 / 1

F, female; **FN**, female neutered; **M**, male; **MN**, male neutered. **NeP score**, neuropathic pain score. **NRS**, numeric rating scale. **OME**, otitis media with effusion - a tentative MRI diagnosis based on T2 hyperintense material in the tympanic bullae. **Syrinx : spinal cord ratio**, ratio of diameters measured where the syrinx was widest on T1-weighted images. **VAS**, visual analogue scale. **VRS**, verbal rating scale. * One owner was not able to rate the dog's quality of life.

Table 2 Pregabalin's effect on secondary outcomes.

Pregabalin's effect on secondary outcomes was assessed by the owner and the PI. A generalised linear mixed model including treatment (pregabalin or placebo), treatment period and follow-up visit number was used to estimate the mean treatment effect in treatment period one compared to baseline values.

	Owner-assessed			PI-assessed		
	Baseline mean	**Treatment effect (CI_{95})**	***P***	**Baseline mean**	**Treatment effect (CI_{95})**	***P***
Scratching intensity						
NRS, 0-10	4.0	- 2.7 (-4.4, -1.0)	0.003	5.2	- 2.7 (-4.2, -1.1)	0.0016
VAS, 0-100 mm	43	- 33 (-46, -19)	< 0.0001	53	- 29 (-46, -12)	0.001
Pain intensity						
NRS, 0-10	2.6	-2.1 (-4.3, 0.05)	0.056	4.2	- 4.1 (-5.6, -2.6)	< 0.0001
VAS, 0-100 mm	34	- 24 (-42, -6)	0.01	44	- 44 (-59, -29)	< 0.0001

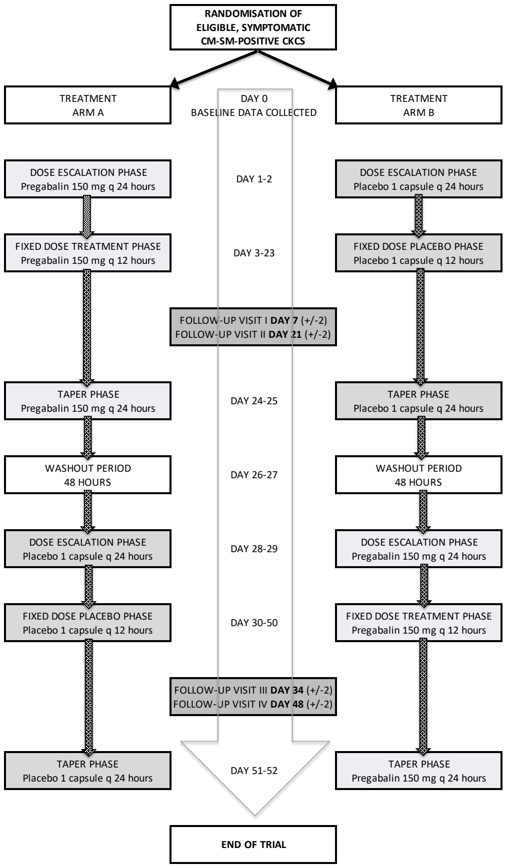
NRS, numeric rating scale. **VAS**, visual analogue scale.

Table 3 Side effects and adverse events.

The side effects and adverse events were reported by the owners at a total of 47 follow-up visits, hereof 23 visits during treatment with pregabalin due to one dropout and 24 visits during the placebo period. The owners were asked a series of closed and semi-open questions addressing **activity level** (normal / reduced / hyperactive), **aggression** (towards other people / animals, yes / no), **appetite** (normal / increased / decreased), **ataxia** (yes / no), **behavioral changes** (yes / no – if yes, please describe), **fecal score** (normal / hard / moist / watery), **food intake** (normal / increased / decreased), **sleeping pattern** (normal / reduced sleeping activity / somnolence), **vomiting** (yes / no), **water intake** (normal / increased / decreased) and details on their dog's **scratching phenotype** (intensity compared to baseline, anatomical localisation, phantom scratching yes/no) and if the dog was **chewing paws** (yes / no).

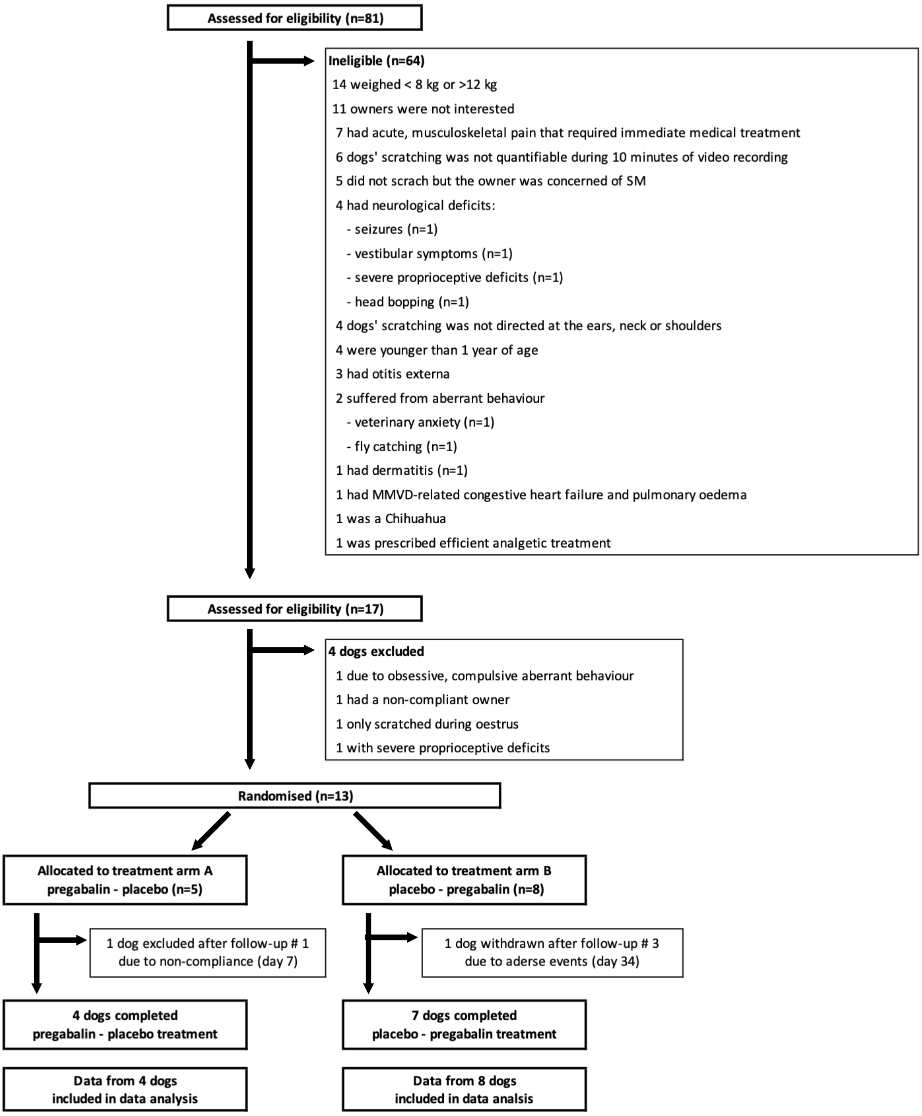
	Treatment	
	PGN	Placebo
Side effects	Total number of side effects in | number of dogs	
Activity level		
Reduced	5 | 4	2 | 1
Hyperactive	2 | 2	1 | 1
Aggression	0	0
Ataxia	14 | 9	1 | 1
Fecal texture: moist	1 | 1	1 | 1
Food intake		
Hypophagia	1 | 1	5 | 4
Polyphagia	18 | 9	3 | 2
Paw chewing	0	0
Sleeping pattern: somnolence	1 | 1	0
Vomiting	0	0
Water intake		
Reduced	0	1 | 1
Increased	4 | 4	1 | 1
Serious adverse events		
Death	0	0
Hospital admission	0	0
Other	0	0

Figure 1



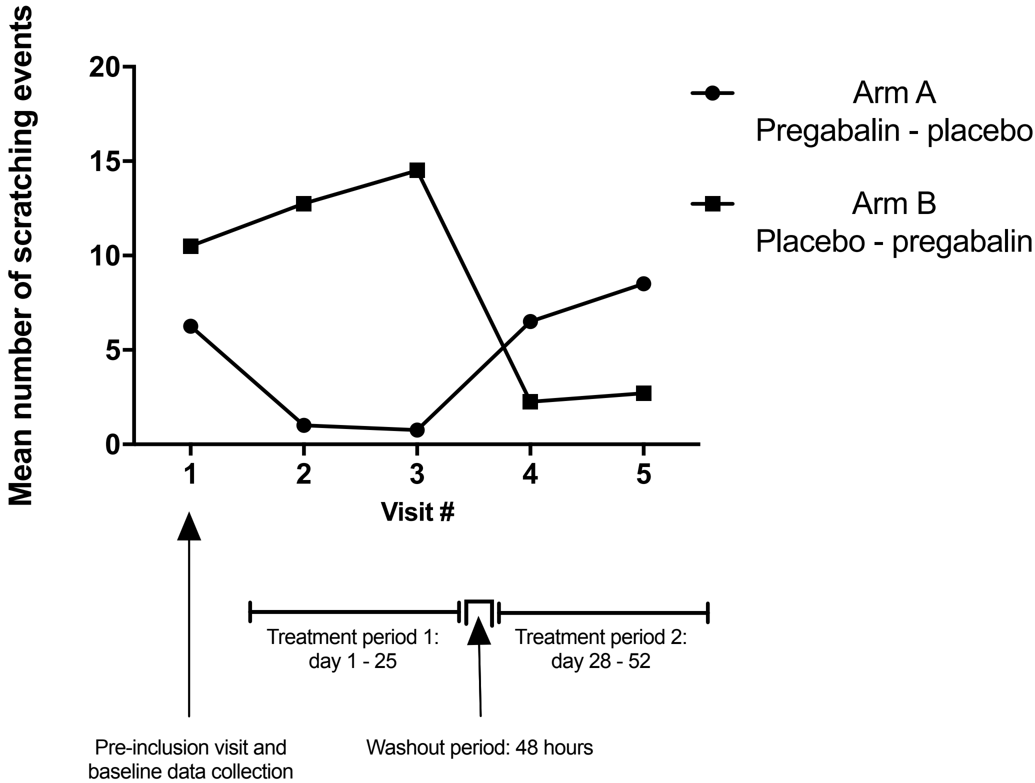
Trial design The trial was designed as a two-treatment two-period crossover study. Eligible Cavalier King Charles Spaniels expressing clinical signs consistent with SM-related central neuropathic pain were randomised to treatment arm A (treatment sequence pregabalin - placebo) or treatment arm B (placebo - pregabalin) after baseline data collection. Each treatment period was of 25 days duration separate by a 48-hour washout period. During the dose escalation and taper phase, 150 mg of pregabalin was administered q 24 hours. In the fixed dose treatment phase, 150 pregabalin was administered q 12 hours. Primary and secondary outcomes were assessed at baseline collection and at follow-up visits I-IV (two visits in each treatment period).

Figure 2



Trial profile Recruitment was initiated in January 2017. Overall, 81 owners and veterinarians responded to the recruitment notices. Of the 81 enquiries, seventeen potential cases (21%) were continuously enrolled in pre-inclusion visits, and 13 dogs were included in the study between March 2017 and June 2018.

Figure 3



Pregabalin's effect on the clinical signs of central neuropathic pain in dogs with syringomyelia
The number of scratching events during ten minutes of continuous, physical activity was quantified after video documentation at baseline and follow-up visits. Included dogs were allocated to treatment arm A (treatment sequence pregabalin - placebo) and treatment arm B (treatment sequence placebo - pregabalin) after **visit 1** (pre-inclusion visit). **Visit 2 and 3** on the x-axis refers to follow-up visits in treatment period one, and **visit 4 and 5** refers to follow-up visits in treatment period two. The two treatment periods were separated by a 48-hour washout period.

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